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Systemic Administration of a Phosphorothioate Oligonucleotide with a Sequence Complementary to p53 for Acute Myelogenous Leukemia and Myelodysplastic Syndrome: Initial Results of a Phase I Trial

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ABSTRACT

A synthetic phosphorothioate oligonucleotide was administered systemically to five patients with either relapsed or refractory acute myelogenous leukemia (AML), or myelodysplastic syndrome (MDS). Patients received a 10-day continuous intravenous infusion of this compound, which is complementary to p53 mRNA. No major toxicity attributable to a dose of 0.05 mg/kg/hr was observed. A range of approximately 9 to 18% of the administered dose was recovered in the urine as intact oligonucleotide. Evaluation of malignant cells recovered from bone marrow and peripheral blood at intervals before, during, and after treatment reveals no enhanced growth potential following oligonucleotide administration. Hence, a phosphorothioate oligonucleotide complementary to p53 mRNA can be administered at this dose level to humans without major toxicity. Higher doses need to be evaluated for toxicity and potential clinical efficacy.

INTRODUCTION

A SYNTHETIC OLIGONUCLEOTIDE with sequence complementary to DNA (antigene) or RNA (antisense) that interferes with the processes of transcription or translation provides, in theory, an approach to gene-specific modulation of gene expression that could provide entirely new therapies for cancer (Tidd, 1991; Wickstrom, 1991). A 20-mer phosphorothioate oligonucleotide (Zon and Stec, 1991), OL(1)p53, which is nuclease resistant and complementary to a segment of exon 10 of p53 mRNA, demonstrated a consistent inhibitory effect on the *in vitro* growth and viability of leukemia blast cells from more than 40 patients with acute myelogenous leukemia (AML), with no apparent effect on normal bone marrow (Bayever et al., 1993).

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The mutant form of the phosphoprotein p53 has been associated with a wide variety of human cancers (Levine et al., 1991; Lane, 1992). The studies reported here represent the systemic treatment of five patients with OL(1)p53 as part of a dose-escalating, phase 1 clinical trial for the treatment of patients with refractory or relapsed AML, or myelodysplastic syndrome (MDS).

SUBJECTS AND METHODS

Subjects

Five patients (two female, three male; ages 16–61 years) received a 10-day continuous infusion (0.05 mg/kg/hr) of OL(1)p53 with the approval of the Institutional Review Board of the University of Nebraska Medical Center (Omaha, NE). Standard supportive care for leukemia induction was utilized, including prophylactic allopurinol, broad-spectrum antibiotics, blood product support, and total parenteral nutrition as indicated. Subjects were diagnosed with either AML (French–American–British [FAB] classifications M1–5) or MDS (FAB classifications RAEB and RAEB/T). Patients with AML had either not responded to two cycles of conventional therapy or had relapsed after a previous remission. Patients in first relapse were required to have a duration of first remission of $\leq$6 months, or to have failed one attempt at induction with established regimens. Exclusion criteria were (1) coexpression on leukemic blasts of CD2, 3, 19, and 20, or (2) prior chemotherapy within 4 weeks, or treatment with any growth factor within 3 weeks, or (3) patients eligible to immediately proceed with bone marrow transplantation, and in whom chemotherapy prior to bone marrow transplantation was considered unnecessary.

Clinical laboratory monitoring included (1) peripheral blood counts with differential, platelets and reticulocytes determined 1 day prior to and then daily during OL(1)p53 infusion, (2) a serum chemistry profile with albumin, alkaline phosphatase, aspartate aminotransferase, bilirubin (total), calcium, γ-glutamyltransferase, glucose, lactate dehydrogenase (LDH), magnesium, phosphorus, protein (total), and uric acid determined 1 day prior to and then daily during OL(1)p53 treatment, (3) a renal profile with chloride, CO_2, serum creatinine, potassium, sodium, urea nitrogen, and anion gap determined 1 day prior to and then three times per week, and (4) determination of serum amylase, iron, iron-binding protein, ferritin complement, C3, C4, IgA, IgG, IgM, anti-nuclear antibody, and anti-neutrophil cytoplasm antibody 1 day prior to and then post OL(1)p53 treatment. Bone marrow aspirates for morphology and cytogenetics collected 1 day prior to and then on days 7, 14, and 28 post initiation of OL(1)p53 infusion. In addition, LDH isoenzyme fractions were determined when elevated LDH levels were observed.

Oligonucleotide preparation

OL(1)p53, 5′-d(CCCTGCTCCCCCCTGGCTCC)-3′, was synthesized and purified by Lynx Therapeutics, Inc. (Foster City, CA), using its proprietary chemistry and methods. The required 60-g batch was shown to be 87% full-length 20-mer that was 99.6% thioated (Bergot and Egan, 1992). Sterility and apyrogenicity were assured according to the guidelines of the U.S. Food and Drug Administration.

Urine recovery of OL(1)p53

Urine was collected as 24-hr pooled samples from patients 1 day prior to treatment and each day during OL(1)p53 administration. Urine (250 μl) was then mixed with 50 μl of sterile water and extracted with an equal volume of phenol–chloroform–water (Applied Biosystems, Inc., Foster City, CA). Four microliters of 1 M cadmium chloride was added to 200 μl of the removed aqueous phase of the extraction to a final concentration of 20 mM, which precipitated the oligonucleotide. The supernatant was removed and the precipitate was dissolved in 70 μl of 3.5 M ammonium acetate in water, which was then extracted with 20 vol of *n*-butanol, which caused precipitation of the oligonucleotide. Following removal of the supernatant the

vacuum-dried precipitate was then dissolved in 50 μl of sterile water and a portion analyzed with an Applied Biosystems, Inc. model 230A high performance electrophoretic chromatography (HPEC). The quantity of OL(1)p53 was determined from the area under the curve measured at 260 nm by a Spectra-Physics SP4290 integrator (Spectra-Physics, San Jose, CA) and interpolation from a standard curve generated with 110 to 740 ng of OL(1)p53 (r^2 = 0.99).

Peripheral blood culture

Suspension culture. Leukemic blast cells from peripheral blood were isolated by Ficoll-Hypaque separation and depleted of T cells by sheep erythrocyte rosetting. The suspension cultures were initiated using a previously described technique (Lange et al., 1987; Hoang and McCulloch, 1985). Viable cell counts were determined by trypan blue exclusion at 3-day intervals and cells were given fresh medium as needed.

Colony-forming units (leukemia) (CFU-L): Enriched AML blast cells were plated in methylcellulose for the primary plating (Lange et al., 1987). A fraction of the cells from day 7 colonies were washed and replated in methylcellulose for secondary plating (Buick et al., 1979).

Long-term bone marrow culture

The mononuclear fraction of bone marrow aspirates from a portion of the sample employed for other studies was obtained by separation on lymphocyte separation medium (LSM) and placed into long-term culture as described previously (Pirruccello et al., 1992). The cultures were demidepopulated weekly, the supernatant cellularity determined, and cytospins prepared for morphological observation. Selected culture samples were processed for cytogenetics by standard techniques (Yunis, 1976), and flow cytometric analysis on a Coulter Elite flow cytometer (Coulter, Hialeah, FL) using Coulter Elite software for comparison to similar results obtained on uncultured and cultured samples taken before therapy was performed.

RESULTS

Characteristics of the five patients at the time of initiation of a 10-day constant infusion of OL(1)p53 are presented in Table 1. Cellular uptake of OL(1)p53 was expected to be slow (Iversen, 1992), therefore constant infusion was selected as an approach to maximizing cellular bioavailability. In addition, constant infusion with no loading dose allowed time to observe toxic responses for several days before steady state plasma concentrations were achieved (Iversen, 1992).

TABLE 1. PATIENT CHARACTERISTICS AT TIME OF INFUSION

			Total white blood cell count (per mm³)		*Absolute neutrophil count (per mm³)*		*Absolute blast count (per mm³)*	
Patient	*Sex/age*	*FAB*[a]	*Day 0*	*Day 10*	*Day 0*	*Day 10*	*Day 0*	*Day 10*
1	M/19	M2	30,700	66,900	1,228	1,338	27,016	57,534
2[b]	F/51	M1	58,700	ND[c]	0	ND	56,352	ND
3	M/16	M2	6,000	52,600	0	0	5,640	51,548
4	M/61	RAEB/M1[d]	4,800	4,700	1,392	1,269	0	0
5	F/46	RAEB/T	19,500	27,500	195	0	18,330	26,400

[a] French–American–British classification.
[b] Infusion stopped on day 6 because of an increase in serum aspartate aminotransferase and alkaline phosphatase.
[c] ND, Not determined.
[d] Initial presentation MDS (RAEB); AML (M1) at time of infusion.

Toxicity

Details of the toxicity according to the Common Toxicity Criteria of the National Cancer Institute (Bethesda, MD) are reported in Table 2 (values range from 0, which indicates no toxicity, to 5, which indicates the most severe toxicity). No unexpected toxicity was observed during infusion and for 4 weeks following the initiation of infusion. Toxicities encountered were associated with the underlying disease and secondary to the severe neutropenia and leukocytosis. An increased incidence of infection and abnormal granulocyte, lymphocyte, platelet, and hemoglobin measurements were observed. Infusion of patient 2 was stopped on day 6 because of an increase in serum aspartate aminotransferase and alkaline phosphatase.

TABLE 2. COMMON TOXICITY CRITERIA[a]

		Patient number				
System	*Measurement*	*1*	*2*	*3*	*4*	*5*
Lung	Pulmonary function	0	1	0	0	0
Heart	Dysrhythmia	0	0	0	0	0
	Ejection fraction	0	0	0	0	0
	Ischemia	0	0	0	0	0
	Pericardial	0	0	0	0	0
	Blood pressure—hypertension	0	1	0	0	0
	Blood pressure—hypotension	0	0	0	0	0
Skin		0	0	0	1	0
Allergy		0	0	0	0	0
Phlebitis		0	0	0	0	0
Alopecia		0	0	0	0	0
Weight		0	1	0	0	0
Leukopenia	White blood cells	0	0	0	0	0
	Granulocytes	4	4	4	2	4
	Lymphocytes	3	4	4	0	0
Thrombocytopenia	Platelets	4	4	4	3	4
Anemia	Hemoglobin	2	3	3	4	2
Hemorrhage		0	0	0	1	0
Infection		2	3	3	0	0
Genitourinary	Creatinine	0	0	1	0	0
	Proteinuria	0	1	0	0	0
	Hematuria	1	1	1	0	0
	Blood urea nitrogen	0	0	1	0	0
Gastrointestinal	Nausea	1	1	2	0	0
	Vomiting	1	0	0	0	0
	Diarrhea	0	0	0	0	0
	Stomatitis	0	0	0	0	0
Liver	Bilirubin	0	1	0	0	1
	Transaminases	0	3	0	0	1
	Alkaline phosphatase	0	3	0	0	0
	Liver—clinical	0	0	0	0	0
Neurological		0	0	0	0	0
Metabolic	Hyperglycemia	0	3	1	1	1
	Hypoglycemia	0	0	0	0	0
	Amylase	0	0	0	0	0
	Hypercalcemia	0	0	0	0	0
	Hypocalcemia	1	0	0	0	0
	Hypomagnesemia	0	0	0	0	0
Coagulation	Fibrinogen	0	0	0	0	0
	Prothrombin time	1	0	0	0	0
	Partial thromboplastin time	1	0	0	0	0

[a]Of the National Cancer Institute.

Pharmacokinetics

Doses of OL(1)p53 administered to each patient are shown in Table 3. The oligonucleotide recovered in the urine was between 30 and 62% of the administered dose within the 10-day infusion (Table 3). Greater than 30% of the OL(1)p53 recovered in urine was apparently full-length material, based on HPEC elution time. Metabolic products were observed both as apparently smaller, faster eluted degradation products and apparently larger, slower eluted metabolites, possibly conjugates. Estimates of half-life ranged from 4.9 to 14.7 days, a wide range, which is not surprising given these patients represent ages from 16 to 61 years, both male and female, with widely differing clinical histories, and white blood cell counts ranging from 4800 to 66,900 at the time OL(1)p53 infusion was started. The plasma concentrations ranged from 2.1 μg/ml (0.32 μM) to 6.4 μg/ml (0.97 μM). Ongoing efforts are in progress to characterize these metabolic products and establish plasma concentrations.

Clinical effects

No clinical effects were noted on the natural history of the underlying disease following infusion of this dose of OL(1)p53 (Table 1), nor was an exacerbation of *in vitro* cell growth of leukemic blasts in bone marrow and peripheral blood cultures noted (Table 4). Bone marrow from routine aspirates was studied in long-term culture and the cumulative cellularity of each sample was measured over at least a 6-week interval. All the bone marrow samples obtained following initiation of infusion had lower production of cells compared to the cultured pretreatment bone marrow, with one exception. For patient 5, cell production was greater from the sample obtained 7 days after the start of infusion. However, the samples obtained from this patient at later times showed a slightly lower cell production, similar to the observations on the other patients. Flow cytometry showed that the phenotypes of the cultured cells were similar to those of the original largely leukemic, pretreatment samples. Cytogenetic analysis showed that metaphases of cells that grew in culture were either all leukemic cells with a clonal abnormality matching the patient's original sample, or mostly leukemic cells with an occasional normal cell. Peripheral blood samples from those patients with sufficient cell counts were also assayed for leukemic blast growth in suspension and semisolid colony cultures. Table 4 shows decreased cell growth in suspension cultures on succeeding days of oligonucleotide infusion. In patients 3 and 5, cell numbers increased slightly after day 7, but in each case they never returned to

TABLE 3. OL(1)p53 DOSE AND PRELIMINARY PHARMACOKINETIC DATA

Patient	*Dose/day (mg)*	*Total dose (mg)*	*Percentage cumulative urinary excretion*[a]	*Half-life*[b] *(days)*	*Cp*[c] *(μg/ml)*
1	69.0	690	62 (10)	4.9	2.1[d]
2[e]	86.4	500	47 (7)	ND[f]	ND
3	74.4	744	30 (10)	ND	ND
4	84.0	840	57 (10); 63 (13)	14.7	6.4
5	81.0	810	42 (10); 55 (12)	5.4	2.3

[a] The concentration in the urine was determined as described in Subjects and Methods. The total amount excreted was determined by multiplying concentration times total urine volume. The numbers in parentheses indicate the day after the initiation of infusion. In patients 4 and 5, recovery after the end of the infusion is reported as the second set of values. The variation in measurements (three to six replicates) falls within a 10% range.

[b] Half-life is determined from the rate of excretion (slope of ln[mg in urine] over time in days) beginning at the end of the infusion.

[c] Cp is the maximum predicted concentration in the plasma, based on the elimination rate in the urine following cessation of infusion, hence an elimination rate (an overestimate of the real plasma concentration).

[d] Patient 1 plasma concentration was determined by ^{32}P-postlabeling with T4 polynucleotide kinase, separation by 20% polyacrylamide gel, and quantitation by autoradiography.

[e] Infusion stopped on day 6.

[f] ND, Not determined.

TABLE 4. CUMULATIVE CELLULARITIES PRODUCED BY BONE MARROW OVER 6-WEEK PERIOD IN LONG-TERM CULTURE AND GROWTH OF LEUKEMIC BLASTS FROM PERIPHERAL BLOOD

	Bone marrow ($\times 10^6$)				*Peripheral blood*					
					Day 0		*Day 7*		*Day 11*	
Patient	*Day 0*	*Day 7*	*Day 14*	*Day 28*	*Suspension ($\times 10^5$)*[a]	*Colonies*[b]	*Suspension ($\times 10^5$)*	*Colonies*	*Suspension ($\times 10^5$)*	*Colonies*
1	ND[c]	ND	ND	ND	5.7 ± 0.5	ND	2.1 ± 0.2	ND	0	ND
2[d]	34	26	ND	ND	12.7 ± 0.5	ND	5.7 ± 0.3	ND	ND	ND
3	63	33	27	ND	10.2 ± 0.8	20 ± 3	3.3 ± 0.3	10 ± 3	5.9 ± 0.9 3.4 ± 0.4[e]	2 ± 2
4[f]	15	7	9	3	ND	ND	ND	ND	ND	ND
5	31[g]	54[g]	29[g]	28[g]	7.3 ± 0.2	4 ± 3	3.3 ± 0.4	0	2.6 ± 0.4 4.7 ± 0.4[e]	0 0[e]

[a] Viable cells counted on day 9 of culture, mean ± standard deviation of triplicate cultures.
[b] Colonies from secondary replating assays, mean ± standard deviation of triplicate cultures.
[c] ND, Not determined.
[d] OL(1)p53 administration stopped on day 6.
[e] Culture from day 30 after initiation of OL(1)p53 infusion.
[f] Insufficient peripheral blasts for culture.
[g] The majority of cells observed appeared morphologically to be normal histiocytes.

pretreatment levels. Similarly, fewer colonies were generated following secondary plating from samples obtained on succeeding days of oligonucleotide administration.

DISCUSSION

A 10-day intravenous infusion of a 20-base phosphorothioate oligonucleotide with sequence complementary to p53 mRNA into five patients with either AML or MDS failed to produce unexpected toxicity either clinically or in the *in vitro* growth of leukemic blast cells from bone marrow and peripheral blood. The major toxicities observed were due to the underlying disease, specifically in blood counts and infection. The increase in aspartate aminotransaminase and alkaline phosphatase in patient 2 by day 6 of the infusion was not seen in the other four patients. Either the leukocytosis ($>100 \times 10^3$) or the concomitant therapeutic intervention with hydroxyurea might have contributed to this finding.

The recovery of up to approximately 60% of OL(1)p53 in urine during the 10-day infusion is consistent with observations from preclinical studies in the rat (our unpublished observations, 1993) and monkey (Cornish et al., 1993). In addition, approximately 30% of the material observed in the urine appeared by preliminary HPEC analysis to be unchanged OL(1)p53, which is also consistent with preclinical observations in rat (our unpublished observations, 1993) and monkey (Cornish et al., 1993). Hence, 30% of between 30 and 60%, that is, 9 to 18%, of the administered OL(1)p53 retained its integrity as bioavailable, potentially active compound.

The bone marrow samples obtained following infusion, with the exception of one sample in one patient, showed a lower cumulative cell production in long-term cultures. Because the cells responsible for initiating long-term cultures are believed to be relatively primitive hematopoietic stem cells, these results suggest the OL(1)p53 may have inhibited the production of progeny by these cells. Alternatively, OL(1)p53 may have reduced the number of cells with clonal ability, or a combination of these effects. Although the cultured cells obtained from patient 5 showing a minimal response appeared to be normal histiocytes, the other cultures in which cell production post-OL(1)p53 infusion was reduced contained largely or entirely leukemic blasts cells. This implies that the OL(1)p53 infusion may have inhibited leukemic cell growth. Further, there was evidence

that the effect apparently persisted after the infusion was complete. If true, this is compatible with the hypothesis that primitive cells (which are slowly turning over even in leukemia) that may have incorporated OL(1)p53 may not be called on to proliferate *in vivo* or in culture for a considerable period of time. However, the cause of apparent decreased cell production remains unknown at this time.

Infusion of OL(1)p53 resulted in an apparent decrease in cell growth of peripheral blood leukemic blasts *in vitro* in suspension and colony-forming cultures. The finding that colonies from secondary plating were affected in patients 3 and 5, in which this assay could be performed, is interesting. A variety of evidence supports a model of AML suggesting that despite the typically uniform appearance of leukemic blasts, they are organized like a normal myelopoietic lineage (McCulloch and Till, 1981; McCulloch et al., 1988). The small proportion of AML blasts (<1%) that are stem cells are measured by secondary plating efficiency, which is an indication of their capacity for self-renewal. There is a strong negative correlation between the secondary plating efficiency for a particular AML blast population and the response of the patient to chemotherapy (Curtis et al., 1984).

In summary, systemic intravenous administration of OL(1)p5 at 0.05 mg/kg/hr for 10 days is not toxic, nor does it lead to enhanced growth of malignant cells. Current investigations focus on evaluation of the toxicity of OL(1)p53 at higher doses. Potential clinical responses will also be evaluated at these higher doses. The presently reported observations of safety and favorable pharmacokinetics support the further investigations of phosphorothioate oligonucleotides as potential gene-specific therapeutic agents.

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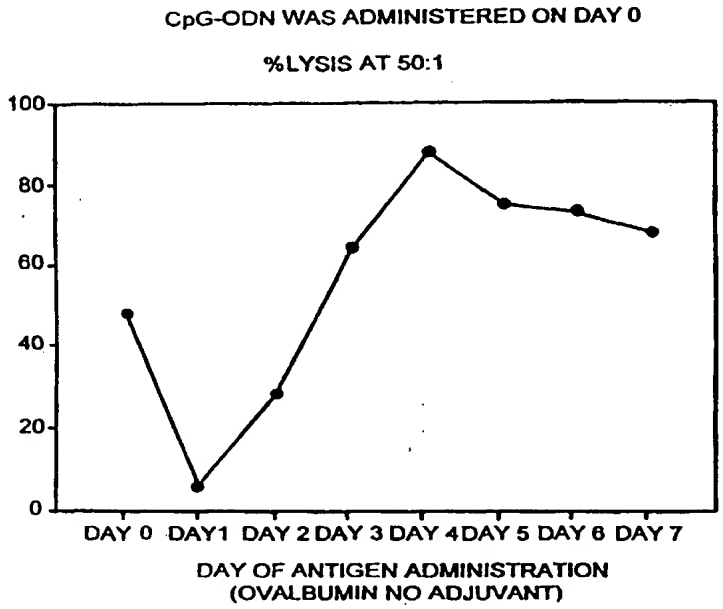

(57) Abstract

The invention relates to methods for regulating hematopoiesis using CpG containing oligonucleotides. In particular, the invention relates to methods of treating thrombopoiesis and anemia by regulating hematopoiesis. The invention also relates to method of regulating immune system remodelling by administering CpG oligonucleotides to control hematopoiesis.

METHODS FOR REGULATING HEMATOPOIESIS USING CpG-OLIGONUCLEOTIDES

Field Of The Invention

The present invention relates to methods for regulating hematopoiesis using CpG containing oligonucleotides. In particular, the invention relates to methods of treating thrombopoiesis and anemia by regulating hematopoiesis. The invention also relates to methods of regulating immune system remodeling by administering CpG oligonucleotides to manipulate hematopoiesis.

Background Of The Invention

Radiation or chemotherapeutic treatment produces severe reversible thrombocytopenia, anemia and neutropenia. The depletion of hematopoietic precursors in the bone marrow (BM) associated with chemotherapy and irradiation result in hemorrhagic and infectious complications. Severe suppression of the hematopoietic system is a major factor in limiting chemotherapy use and dose escalation. A number of hematopoietic cytokines are currently in clinical trials as treatments to prevent or reduce such complications.

Hematopoietic development is considered to be regulated by two categories of factors. One category includes colony-stimulating factors (CSFs), which promote colony formation and proliferation of cells of various lineages. Another is potentiators, which potentiate maturation or differentiation. For example, Megakaryocyte-CSFs (Meg-CSFs) reportedly include IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF), and Megakaryocyte potentiators (Meg-Pot) reportedly include IL-6, IL-7, IL-11, erythropoietin (EPO) and leukemia inhibitory factor (LIF). Platelet production is a terminal phenomenon in the development of megakaryocytes *in vivo*. Thrombopoietin (TPO) was reported to posses both Meg-CSF and Meg-Pot.

In early days of interferon (IFN) research Isaacs et al. postulated that foreign DNA induces IFN. Rotem, Z et al. (1963) Nature 197:564-566; Jensen, KE et al. (1963) Nature 200:433-434. Later it was discovered that synthetic double-stranded RNA was able to induce IFN and to activate both natural killer (NK) cells and macrophages. Field, AK et al. (1967) Proc Natl Acad Sci USA 58:1004-1010. Subsequently, Yamamoto, Tokunaga and colleagues discovered immunostimulatory DNA by a series of studies originally aimed at analyzing

374:546-549; Bird, AP (1986) Nature 321:209-213. The realization that these sequences are under-represented in vertebrate DNA offers an explanation for several biological observations in the context of non-self pattern recognition by the immune system.

CpG DNA induced *in vivo* immune responses

The immunogenicity of proteinaceous natural and recombinant purified antigens is poor unless aided by adjuvants. Because of the apparent recognition and response to foreign DNA by the immune system, the potential of CpG DNA to serve as an adjuvant was previously tested. Mice were challenged subcutaneously with liposome-encapsulated ovalbumin (used as antigen) and CpG-ODN (used as adjuvant) using a protocol described by Lipford et al. Lipford, GB et al. (1997) Eur J Immunol 27:3420-3426. The mice which were co-administered CpG-ODN developed strong peptide-specific cytotoxic T lymphocyte (CTL) activity in the draining lymph nodes (LNs). Furthermore, not only was the antibody response augmented, but CpG-ODN switched the isotype pattern to a Th1-type profile, in that antigen-specific IgG2a became dominant. Lipford, GB et al. (1997) Eur J Immunol 27:3420-3426. This pattern of strong CTL induction and Th1 biasing in the antibody repertoire has been extended to other protein antigens. Subsequently, it has been found that the use of liposome as antigen carriers is not necessary for CTL induction. This observation was unexpected because typically soluble protein antigens can not enter the major histocompatibility complex (MHC) class I presentation pathway and therefore can not be presented to precursor CTL by antigen-presenting cells (APCs).

The Th1 biasing of CpG DNA when co-administered with protein antigen has now been fully documented. Roman et al. demonstrated the dominance of antigen-specific IgG2a induction when using as the adjuvant either CpG-ODN, plasmid DNA containing CpG motifs, or bacterial DNA. The Th1-promoting adjuvanticity of CpG-ODN may be useful for the redirection to protective, or even curative, responses in Th2-driven disorders. A model is the CpG-ODN modulation of Th2 driven airway inflammation in a murine model of asthma induced with *Schistosoma mansoni* eggs. Airway eosinophilia, Th2 cytokine induction, IgE production and bronchial hyperreactivity were prevented by CpG-ODN co-administration with egg sensitization. Additionally, egg sensitized mice treated at day 7 post sensitization with CpG-ODN and antigen were protected from airway eosinophilia. Similar results were obtained in an infection model for the redirection of Th2 responses to protective Th1

McIntyre et al. unexpectedly observed that the control p65-sense, but not the p65-antisense, oligonucleotides caused massive splenomegaly in mice. McIntyre, KW et al. (1993) Antisense Res Dev 3:309-322. In this study they demonstrated a sequence-specific stimulation of splenic cell proliferation, both *in vivo* and *in vitro*, by treatment with p65-sense oligonucleotides. Cells expanded by this treatment were primarily B-220+, sIg+ B cells. The secretion of immunoglobulins by the p65-sense oligonucleotide-treated splenocytes was also enhanced. In addition, the p65-sense-treated splenocytes, but not several other cell lines, showed an upregulation of NF-κB-like activity in the nuclear extracts, an effect not dependent on new protein or RNA synthesis. Zhao et al. concluded that phosphorothioate ODN induce splenomegaly due to B cell proliferation. Zhao, Q et al. (1996) Biochem Pharmacol 51:173-182. In a follow-up study Zhao et al. found administration of the 27-mer-phosphorothioate oligonucleotide into mice resulted in splenomegaly and an increase in IgM production 48 hr post-administration. Zhao, Q et al. (1996) Biochem Pharmacol 52:1537-1544.

Agrawal et al. evaluated the *in vivo* toxicological effects of phosphorothioate oligodeoxynucleotides (PS oligo). Agrawal, S et al. (1997) Antisense Nucleic Acid Drug Dev 7:575-584. Oligodeoxynucleotides were administrated intravenously to male and female rats at doses of 3, 10, and 30 mg/kg/day for 14 days. Rats were killed on day 15, blood samples were collected for hematology and clinical chemistry determinations, and tissues, including lymph nodes, spleens, livers, and kidneys, were subjected to pathologic examinations. The toxicity profiles of four oligodeoxynucleotides were very similar, but differed in magnitude. Alterations in hematology parameters included thrombocytopenia, anemia, and neutropenia. Dose-dependent enlargements of spleen, liver, and kidney were observed. Pathologic studies showed a generalized hyperplasia of the reticuloendothelial system in the tissues examined.

Krieg et al. reported that bacterial DNA and synthetic oligodeoxynucleotides containing unmethylated CpG dinucleotides induce murine B cells to proliferate and secrete immunoglobulin *in vitro* and *in vivo*. Krieg, AM et al. (1995) Nature 374:546-549. This activation is enhanced by simultaneous signals delivered through the antigen receptor. Optimal B cell activation requires a DNA motif in which an unmethylated CpG dinucleotide is flanked by two 5' purines and two 3' pyrimidines. Oligodeoxynucleotides containing this CpG motif induce more than 95% of all spleen B cells to enter the cell cycle. In a study by Monteith et al., treatment of rodents with phosphorothioate oligodeoxynucleotides induced a form of immune stimulation characterized by splenomegaly, lymphoid hyperplasia,

dose of the oligonucleotides. Serum release of IL-1, IL-6, IL-12 and TNF-α was also confirmed by Lipford et al. Lipford, GB et al. (1997) Eur J Immunol 27:2340-2344.

Hendrzak and Brunda demonstrated that administration of IL-12 in mice caused thrombocytopenia, splenomegaly, and mononuclear cell infiltration, an explanation for the splenomegaly. Hendrzak and Brunda (1995) Lab Invest 72:619-637. IL-12 has been shown to be released in response to CpG-ODN and is an inducer of IFN-γ. Control of intracellular bacterial infections requires IFN-γ both for establishing a Th1 T-cell response and for activating macrophages to kill the bacteria. Murray et al. observed that exposure of mice deficient in IFN-γ to mycobacterial infection produces an immune response characterized by a Th2 T-cell phenotype, florid bacterial growth, and death. Murray, PJ et al. (1998) Blood 91:2914-2924. They reported that IFN-γ-deficient mice infected with mycobacteria also undergo a dramatic remodeling of the hematopoietic system. Myeloid cell proliferation proceeds unchecked throughout the course of mycobacterial infection, resulting in a transition to extramedullary hematopoiesis. The splenic architecture of infected IFN-γ-deficient mice is completely effaced by expansion of macrophages, granulocytes, and extramedullary hematopoietic tissue. These features coincide with splenomegaly, an increase in splenic myeloid colony-forming activity, and marked granulocytosis in the peripheral blood. Systemic levels of cytokines are elevated, particularly IL-6 and granulocyte colony-stimulating factor (G-CSF). These results suggest that in addition to its central rôle in cellular immunity, IFN-γ may be a key cytokine in the coordinate regulation of immune effector cells and myelopoiesis. Several studies have noted the *in vitro* inhibition of colony forming units by IFN-γ. Thus according to the prior art strong Th1 responses as characterized by IFN-γ release may be inhibitory for hematopoiesis events.

Although it has been believed that IL-3/GM-CSF/IL-5 (Th0 and Th2 cytokines) produced by activated T cells play a major role in expansion of hematopoietic cells in emergency, results indicate that the entire function of IL-3/GM-CSF/IL-5 is dispensable for hematopoiesis in emergency as well as in the steady state. Thus, there must be an alternative mechanism to produce blood cells in both situations. IL-13, a recently identified Th2 cytokine, shares some, but not all, IL-4 functions, including inhibition of monocyte and macrophage activation, stimulation of human B cells, and induction of growth and differentiation of mouse bone marrow cells *in vitro*. Lai et al. tested the *in vivo* effects of recombinant mouse IL-13 (rIL-13) from stably transfected, high expressing BW5147

specific immune response. The method is based on the finding that a CpG oligonucleotide can be used to induce remodeling of the immune system by regulating hematopoiesis. After a CpG oligonucleotide and antigen are administered together to a subject an initial immune response occurs. It has been discovered according to the invention that this initial immune response declines rapidly and a new immune response develops after approximately 48 hours. Unexpectedly, when antigen is administered 48 hours or more after the administration of CpG an antigen specific immune response will be mounted to the antigen. This immune response is due to a repopulation of lymph nodes and/or spleen with primed immune cells.

Thus, in one aspect the invention is a method for inducing an antigen-specific immune response by administering to a subject an oligonucleotide, having a sequence including at least the following formula:

$$5' X_1CGX_2 3'$$

wherein the oligonucleotide includes at least 8 nucleotides wherein C and G are unmethylated and wherein X_1 and X_2 are nucleotides, and exposing the subject to an antigen at least 3 days after the oligonucleotide is administered to the subject to produce an antigen-specific immune response.

The subject may be exposed to the antigen at least 48 hours after the CpG oligonucleotide is administered to the subject. It has been discovered that immune system remodeling begins to occur within 48 hours of CpG administration. It has also been discovered that the primed immune cells are still capable of responding to antigen even 30 days after CpG administration. In one embodiment the antigen is administered at least 4 days after the oligonucleotide is administered to the subject. In another embodiment the antigen is administered at least 7 days after the oligonucleotide is administered to the subject. In another embodiment the antigen is administered at least 15 days after the oligonucleotide is administered to the subject. In yet another embodiment the antigen is administered at least 30 days after the oligonucleotide is administered to the subject.

The antigen may be any type of antigen known in the art. For instance, in some embodiments the antigen may be cells, cell extracts, proteins, peptides, polysaccharides, polysaccharide conjugates, lipids, glycolipids, carbohydrate, viral extracts, viruses, bacteria, fungi, parasites, and allergens. In other embodiments the antigen may be a nucleic acid encoding an antigen.

In a preferred embodiment the antigen is an allergen and the method is a method for

According to another aspect the invention is a method of treating a subject at risk of developing thrombocytopenia by administering to a subject at risk of developing thrombocytopenia an oligonucleotide, having a sequence including at least the following formula:

$$5' X_1CGX_2 3'$$

wherein the oligonucleotide includes at least 8 nucleotides wherein C and G are unmethylated and wherein X_1 and X_2 are nucleotides, in an amount effective to prevent a decrease in platelet counts ordinarily expected under platelet-depleting conditions in the subject when the subject is exposed to platelet-depleting conditions.

In one embodiment the oligonucleotide is administered in an amount effective to increase platelet counts in the subject by at least 10,000 platelets per microliter. In another embodiment the oligonucleotide is administered in an amount effective to increase platelet counts in the subject by at least 20,000 platelets per microliter. In yet another embodiment the oligonucleotide is administered to the subject in an amount effective to increase the platelet counts in the subject by 100 percent.

The thrombocytopenia is any type of thrombocytopenia known in the art. In one embodiment the thrombocytopenia is a drug-induced thrombocytopenia. According to another embodiment the thrombocytopenia is due to an autoimmune disorder such as idiopathic thrombocytopenic purpura. In yet another embodiment the thrombocytopenia is a thrombocytopenia resulting from accidental radiation exposure. The thrombocytopenia is a thrombocytopenia resulting from therapeutic radiation exposure in yet another embodiment.

According to another aspect the invention is a method for treating anemia by administering to a subject having anemia an oligonucleotide, having a sequence including at least the following formula:

$$5' X_1CGX_2 3'$$

wherein the oligonucleotide includes at least 8 nucleotides wherein C and G are unmethylated and wherein X_1 and X_2 are nucleotides, in an amount effective to induce erythropoiesis in the subject.

In one embodiment the oligonucleotide is administered in an amount effective to increase erythroblast counts in the subject by at least 10 percent. In another embodiment the oligonucleotide is administered in an amount effective to increase erythroblast counts in the

In another embodiment the CpG oligonucleotide has a sequence including at least the following formula:

$$5' \text{ TCNTX}_1\text{X}_2\text{CGX}_3\text{X}_4 \text{ 3'}$$

wherein X_1, X_2, X_3, and X_4 are nucleotides, N is a nucleic acid sequence composed of from about 0-25 nucleotides.

X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA and X_3X_4 are nucleotides selected from the group consisting of: TpT andCpT in another embodiment.

Each of the limitations of the invention can encompass various embodiments of the invention. It is, therefore, anticipated that each of the limitations of the invention involving any one element or combinations of elements can be included in each aspect of the invention.

Brief Description Of The Drawings

Figure 1 is a graph depicting the kinetics of increased spleen weight induced by CpG-ODN.

Figure 2 is a graph depicting the changes in phenotype of spleen cells after stimulation with CpG-ODN.

Figure 3 is a graph depicting the CpG-ODN induced changes in splenic cell number, number of splenic and BM GM-CFU.

Figure 4 is a graph depicting the dose titration of CpG-ODN.

Figure 5 is a graph depicting the increased number of BFU-E induced by CpG-ODN.

Figure 6 is a graph depicting the determination of spleen colony forming units of normal vs. CpG-ODN induced spleen cells (CFU-S Assay).

Figure 7 is a graph depicting the increased number of CM-CFU and enhanced CTL function after ODN-injection correlates with increased resistance towards lethal listeriosis in sublethally irradiated mice.

Figure 8 is a pair of graphs depicting spleen weights and spleen cell counts 5 days following 5 fluorouracil administration to mice, with or without coadministration of CpG-ODN.

Figure 9 is a graph depicting the splenic T lymphocyte counts on days 4, 7, and 10 following 5 fluorouracil administration to mice, with or without coadministration of CpG-ODN.

Figure 10 is a graph depicting the splenic B lymphocyte counts on days 4, 7, and 10

cells is controlled through the complex interaction of immune factors such as interleukin and CSF. Using these factors the immune system is able to regulate the levels of each of the cellular components in blood in response to physiological changes.

Erythrocytes, leukocytes and platelets are the essential cells of the human hematopoietic system. The primary function of erythrocytes, also known as red blood cells, is to transport hemoglobin, which in turn carries oxygen from the lungs to tissues. Oxygenated hemoglobin gives the erythrocytes a red color. Leukocytes, also referred to as myeloid cells, are a heterogenous group of cells that mediate immune responses and which include granulocytes, including eosinophils, basophils, and neutrophils; monocytes; and T and B lymphocytes. These cells are found predominately in the blood, bone marrow, lymphoid organs and epithelium. Leukocytes are referred to as white blood cells because of a lack of natural pigment which gives the cells a whitish or transparent appearance. Platelets play a role in hemostasis, or the regulation of bleeding.

Many factors are capable of influencing the hematopoietic system causing deficiencies or malignancies of particular types of blood cells. Disorders of the hematopoietic system vary depending on the factor causing the disorder as well as the cell type affected.

The invention involves the discovery that CpG containing oligonucleotides can regulate hematopoieses to inhibit loss of blood cells in response to physiological disorders caused by genetic abnormalities, environmental factors or medical therapies. In another aspect the invention involves the discovery that hematopoiesis can be manipulated using CpG oligonucleotides to induce immune system remodeling in order to stimulate an antigen specific immune response.

In one aspect the invention is a method for inducing immune system remodeling. The process of immune system remodeling is based on the generation of immune cells in response to a stimuli in preparation for generating a strong antigen specific immune response. The stimulus is a CpG oligonucleotide. It has been discovered according to the invention that when a CpG oligonucleotide is administered to a subject, after an initial delay, the immune system of the subject undergoes a repopulation event to produce a population of immune cells which are primed to generate an antigen specific response. This renewed population of cells remains in the body for an extensive period of time. When the primed cells encounter antigen the cells respond to the antigen by producing an antigen specific immune response. In fact the

inducing an antigen-specific immune response, by administering to a subject an oligonucleotide, having a sequence including at least the following formula:

$$5' X_1CGX_2 3'$$

wherein the oligonucleotide includes at least 8 nucleotides wherein C and G are unmethylated and wherein X_1 and X_2 are nucleotides, and exposing the subject to an antigen at least 3 days after the oligonucleotide is administered to the subject to produce an antigen-specific immune response.

An "antigen" as used herein is a molecule capable of provoking an immune response. Antigens include but are not limited to cells, cell extracts, polysaccharides, polysaccharide conjugates, lipids, glycolipids, carbohydrate, peptides, proteins, viruses, and viral extracts. The term antigen broadly includes any type of molecule which is recognized by a host immune system as being foreign. Antigens include but are not limited to cancer antigens, microbial antigens, and allergens.

The methods of the invention are useful for treating cancer by stimulating an antigen specific immune response against an antigen. A "cancer antigen" as used herein is a compound, such as a peptide, associated with a tumor or cancer cell surface and which is capable of provoking an immune response when expressed on the surface of an antigen presenting cell in the context of an MHC molecule. Cancer antigens can be prepared from cancer cells either by preparing crude extracts of cancer cells, for example, as described in Cohen, et al., 1994, *Cancer Research*, 54:1055, by partially purifying the antigens, by recombinant technology, or by de novo synthesis of known antigens. Cancer antigens include antigens that are recombinately an immunogenic portion of or a whole tumor or cancer. Such antigens can be isolated or prepared recombinatly or by any other means known in the art. Cancers or tumors include but are not limited to biliary tract cancer; brain cancer; breast cancer; cervical cancer; choriocarcinoma; colon cancer; endometrial cancer; esophogeal cancer; gastric cancer; intraepithelial neoplasms; lymphomas; liver cancer; lung cancer (e.g. small cell and non-small cell); melanoma; neuroblastomas; oral cancer; ovarian cancer; pancreas cancer; prostate cancer; rectal cancer; sarcomas; skin cancer; testicular cancer; thyroid cancer; and renal cancer, as well as other carcinomas and sarcomas.

The methods of the invention are also useful for treating infectious diseases. An infectious disease, as used herein, is a disease arising from the presence of a foreign microorganism in the body. CpG is used to stimulate an antigen specific immune response

Borelia burgdorferi, *Legionella pneumophilia*, *Mycobacteria sps* (e.g. *M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansaii*, *M. gordonae*), *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus* (viridans group), *Streptococcus faecalis*, *Streptococcus bovis*, *Streptococcus* (anaerobic sps.), *Streptococcus pneumoniae*, pathogenic *Campylobacter sp.*, *Enterococcus sp.*, *Haemophilus influenzae*, *Bacillus antracis*, *corynebacterium diphtheriae*, *corynebacterium sp.*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringers*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Bacteroides sp.*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidium*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israelli*.

Examples of infectious fungi include: *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Chlamydia trachomatis*, *Candida albicans*. Other infectious organisms (i.e., protists) include: *Plasmodium* such as *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium* vivax and *Toxoplasma gondii*.

Other medically relevant microorganisms have been descried extensively in the literature, e.g., see C.G.A Thomas, *Medical Microbiology*, Bailliere Tindall, Great Britain 1983, the entire contents of which is hereby incorporated by reference.

The methods of the invention are also useful for treating allergic diseases. The methods are accomplished in the same way as described above for the tumor immunotherapy and treatment of infectious diseases except that the antigen is specific for an allergen. Currently, allergic diseases are generally treated by the injection of small doses of antigen followed by subsequent increasing dosage of antigen. It is believed that this procedure produces a memory immune response to prevent further allergic reactions. These methods, however, are associated with the risk of side effects such as an allergic response. The methods of the invention avoid these problems.

An "allergen" refers to a substance (antigen) that can induce an allergic or asthmatic response in a susceptible subject. The list of allergens is enormous and can include pollens, insect venoms, animal dander dust, fungal spores and drugs (e.g. penicillin). Examples of natural, animal and plant allergens include but are not limited to proteins specific to the following genuses: *Canine (Canis familiaris)*; *Dermatophagoides* (e.g. *Dermatophagoides*

asthma. Th2 cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines, especially IL-4 and IL-5 are elevated in the airways of asthmatic subjects. These cytokines promote important aspects of the asthmatic inflammatory response, including IgE isotope switching, eosinophil chemotaxis and activation and mast cell growth. Th1 cytokines, especially IFN-γ and IL-12, can suppress the formation of Th2 clones and production of Th2 cytokines. "Asthma" refers to a disorder of the respiratory system characterized by inflammation, narrowing of the airways and increased reactivity of the airways to inhaled agents. Asthma is frequently, although not exclusively associated with atopic or allergic symptoms.

It is believed that the antigen is taken up by an antigen presenting cell (APC) such as a dendritic cell in the repopulated immune system. The APC then processes and presents the antigen on its cell surface to produce a cytotoxic T lymphocyte (CTL) response by interacting with T lymphocytes or an antibody response by interacting with B lymphocytes. Preferably, the antigen is exposed to the immune cells 48 hours after adding CpG. In a more preferred embodiment, the subject's immune cells are exposed to the antigen 60 hours after the CpG. In other embodiments the subject's immune cells are exposed to the antigen at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,1 8, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 days after the CpG.

A "subject" shall mean a human or vertebrate animal including but not limited to a dog, cat, horse, cow, pig, sheep, goat, chicken, primate, e.g., monkey, fish (aquaculture species), e.g. salmon, rat, and mouse.

Although many of the disorders described above relate to human disorders, the invention is also useful for treating other nonhuman vertebrates. Nonhuman vertebrates are also capable of developing cancer, infections, allergies, and asthma. For instance, in addition to the treatment of infectious human diseases, the methods of the invention are useful for treating infections of animals. As used herein, the term "treat" or "treating" when used with respect to an infectious disease refers to a prophylactic treatment which increases the resistance of a subject to infection with a pathogen or, in other words, decreases the likelihood that the subject will become infected with the pathogen. Many vaccines for the treatment of non-human vertebrates are disclosed in Bennett, K. *Compendium of Veterinary Products*, 3rd ed. North American Compendiums, Inc., 1995.

Thus the present invention contemplates the use of CpG oligonucleotides to induce an

Vesicular exanthema of swine virus, San Miguel sea lion virus, Feline picornavirus and Norwalk virus; the family Togaviridae, including the genus Alphavirus (Eastern equine encephalitis virus, Semliki forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavirius (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyvirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi and related viruses); the family Orthomyxoviridae, including the genus Influenza virus (Influenza virus type A, many human subtypes); Swine influenza virus, and Avian and Equine Influenza viruses; influenza type B (many human subtypes), and influenza type C (possible separate genus); the family paramyxoviridae, including the genus Paramyxovirus (Parainfluenza virus type 1, Sendai virus, Hemadsorption virus, Parainfluenza viruses types 2 to 5, Newcastle Disease Virus, Mumps virus), the genus Morbillivirus (Measles virus, subacute sclerosing panencephalitis virus, distemper virus, Rinderpest virus), the genus Pneumovirus (respiratory syncytial virus (RSV), Bovine respiratory syncytial virus and Pneumonia virus of mice); forest virus, Sindbis virus, Chikungunya virus, O'Nyong-Nyong virus, Ross river virus, Venezuelan equine encephalitis virus, Western equine encephalitis virus), the genus Flavirius (Mosquito borne yellow fever virus, Dengue virus, Japanese encephalitis virus, St. Louis encephalitis virus, Murray Valley encephalitis virus, West Nile virus, Kunjin virus, Central European tick borne virus, Far Eastern tick borne virus, Kyasanur forest virus, Louping III virus, Powassan virus, Omsk hemorrhagic fever virus), the genus Rubivirus (Rubella virus), the genus Pestivirus (Mucosal disease virus, Hog cholera virus, Border disease virus); the family Bunyaviridae, including the genus Bunyvirus (Bunyamwera and related viruses, California encephalitis group viruses), the genus Phlebovirus (Sandfly fever Sicilian virus, Rift Valley fever virus), the genus Nairovirus (Crimean-Congo hemorrhagic fever virus, Nairobi sheep disease virus), and the genus Uukuvirus (Uukuniemi

Aviadenovirus (Avian adenoviruses); and non-cultivatable adenoviruses; the family Papoviridae, including the genus Papillomavirus (Human papilloma viruses, bovine papilloma viruses, Shope rabbit papilloma virus, and various pathogenic papilloma viruses of other species), the genus Polyomavirus (polyomavirus, Simian vacuolating agent (SV-40), Rabbit vacuolating agent (RKV), K virus, BK virus, JC virus, and other primate polyoma viruses such as Lymphotrophic papilloma virus); the family Parvoviridae including the genus Adeno-associated viruses, the genus Parvovirus (Feline panleukopenia virus, bovine parvovirus, canine parvovirus, Aleutian mink disease virus, etc). Finally, DNA viruses may include viruses which do not fit into the above families such as Kuru and Creutzfeldt-Jacob disease viruses and chronic infectious neuropathic agents (CHINA virus).

Both gram negative and gram positive bacteria serve as antigens in vertebrate animals. Such gram positive bacteria include, but are not limited to those bacteria discussed above as well as *Pasteurella* species, *Staphylococci* species, and *Streptococcus* species. Gram negative bacteria include, but are not limited to, *Escherichia coli*, *Pseudomonas* species, and *Salmonella* species. *Salmonella* enteritidis is an important pathogen in the commercial layer industry, as ovarian colonization of layers may result in maternally transmitted *Salmonella* in table eggs.

In addition to the use of CpG oligonculeotides to induce an antigen specific immune responses in humans, the methods of the preferred embodiments are particularly well suited for treatment of birds such as hens, chickens, turkeys, ducks, geese, quail, and pheasant. Birds are prime targets for many types of infections including AIDS or immunodeficiency virus.

Hatching birds are exposed to pathogenic microorganisms shortly after birth. Although these birds are initially protected against pathogens by maternal derived antibodies, this protection is only temporary, and the bird's own immature immune system must begin to protect the bird against the pathogens. It is often desirable to prevent infection in young birds when they are most susceptible. It is also desirable to prevent against infection in older birds, especially when the birds are housed in closed quarters, leading to the rapid spread of disease. Thus, it is desirable to administer the CpG oligonucleotide of the invention to birds to enhance an antigen-specific immune response when antigen is present.

An example of a common infection in chickens is chicken infectious anemia virus (CIAV). CIAV was first isolated in Japan in 1979 during an investigation of a Marek's disease vaccination break (Yuasa et al., 1979, Avian Dis. 23:366-385). Since that time, CIAV

Cattle and livestock are also susceptible to infection. Disease which affect these animals can produce severe economic losses, especially amongst cattle. The methods of the invention can be used to protect against infection in livestock, such as cows, horses, pigs, sheep, and goats.

Cows can be infected by bovine viruses. Bovine viral diarrhea virus (BVDV) is a small enveloped positive-stranded RNA virus and is classified, along with hog cholera virus (HOCV) and sheep border disease virus (BDV), in the pestivirus genus. Although, Pestiviruses were previously classified in the Togaviridae family, some studies have suggested their reclassification within the Flaviviridae family along with the flavivirus and hepatitis C virus (HCV) groups (Francki, et al., 1991).

BVDV, which is an important pathogen of cattle can be distinguished, based on cell culture analysis, into cytopathogenic (CP) and noncytopathogenic (NCP) biotypes. The NCP biotype is more widespread although both biotypes can be found in cattle. If a pregnant cow becomes infected with an NCP strain, the cow can give birth to a persistently infected and specifically immunotolerant calf that will spread virus during its lifetime. The persistently infectected cattle can succumb to mucosal disease and both biotypes can then be isolated from the animal. Clinical manifestations can include abortion, teratogenesis, and respiratory problems, mucosal disease and mild diarrhea. In addition, severe thrombocytopenia, associated with herd epidemics, that may result in the death of the animal has been described and strains associated with this disease seem more virulent than the classical BVDVs.

Equine herpesviruses (EHV) comprise a group of antigenically distinct biological agents which cause a variety of infections in horses ranging from subclinical to fatal disease. These include Equine herpesvirus-1 (EHV-1), a ubiquitous pathogen in horses. EHV-1 is associated with epidemics of abortion, respiratory tract disease, and central nervous system disorders. Primary infection of upper respiratory tract of young horses results in a febrile illness which lasts for 8 to 10 days. Immunologically experienced mares may be reinfected via the respiratory tract without disease becoming apparent, so that abortion usually occurs without warning. The neurological syndrome is associated with respiratory disease or abortion and can affect animals of either sex at any age, leading to incoordination, weakness and posterior paralysis (Telford, E. A. R. et al., Virology 189, 304-316, 1992). Other EHV's include EHV-2, or equine cytomegalovirus, EHV-3, equine coital exanthema virus, and EHV-4, previously classified as EHV-1 subtype 2.

have been afflicted with FIP include the lynx and caracal, sand cat, and pallas cat. In domestic cats, the disease occurs predominantly in young animals, although cats of all ages are susceptible. A peak incidence occurs between 6 and 12 months of age. A decline in incidence is noted from 5 to 13 years of age, followed by an increased incidence in cats 14 to 15 years old.

Viral and bacterial diseases in fin-fish, shellfish or other aquatic life forms pose a serious problem for the aquaculture industry. Owing to the high density of animals in the hatchery tanks or enclosed marine farming areas, infectious diseases may eradicate a large proportion of the stock in, for example, a fin-fish, shellfish, or other aquatic life forms facility. Prevention of disease is a more desired remedy to these threats to fish than intervention once the disease is in progress. Vaccination of fish is the only preventative method which may offer long-term protection through immunity. Nucleic acid based vaccinations are described in US Patent No. 5,780,448 issued to Davis.

The fish immune system has many features similar to the mammalian immune system, such as the presence of B cells, T cells, lymphokines, complement, and immunoglobulins. Fish have lymphocyte subclasses with roles that appear similar in many respects to those of the B and T cells of mammals. Vaccines can be administered orally or by immersion or injection.

Aquaculture species include but are not limited to fin-fish, shellfish, and other aquatic animals. Fin-fish include all vertebrate fish, which may be bony or cartilaginous fish, such as, for example, salmonids, carp, catfish, yellowtail, seabream, and seabass. Salmonids are a family of fin-fish which include trout (including rainbow trout), salmon, and Arctic char. Examples of shellfish include, but are not limited to, clams, lobster, shrimp, crab, and oysters. Other cultured aquatic animals include, but are not limited to eels, squid, and octopi.

Polypeptides of viral aquaculture pathogens include but are not limited to glycoprotein (G) or nucleoprotein (N) of viral hemorrhagic septicemia virus (VHSV); G or N proteins of infectious hematopoietic necrosis virus (IHNV); VP1, VP2, VP3 or N structural proteins of infectious pancreatic necrosis virus (IPNV); G protein of spring viremia of carp (SVC); and a membrane-associated protein, tegumin or capsid protein or glycoprotein of channel catfish virus (CCV).

Polypeptides of bacterial pathogens include but are not limited to an iron-regulated outer membrane protein, (IROMP), an outer membrane protein (OMP), and an A-protein of

soldiers or civilians at risk of exposure to biowarfare.

Thus, the invention contemplates scheduled administration of CpG oligonucleotides. The oligonucleotides may be administered to a subject on a weekly or monthly basis. When a subject is at risk of exposure to an antigen or antigens the CpG may be administered on a regular basis to maintain a primed immune system that will recognize the antigen immediately upon exposure and produce an antigen specific immune reponse. A subject at risk of exposure to an antigen is any subject who has a high probability of being exposed to an antigen and of developing an immune response to the antigen. If the antigen is an allergen and the subject develops allergic responses to that particular antigen and the subject is exposed to the antigen, i.e., during pollen season, then that subject is at risk of exposure to the antigen. If such a subject is administered a CpG oligonucleotide on a monthly basis then they will maintain a primed set of immune cells which are capable of recognizing and reacting to an antigen.

A subject at risk of developing a cancer can also be treated according to the methods of the invention, by passive or active exposure to antigen following CpG. A subject at risk of developing a cancer is one who is who has a high probability of developing cancer. These subjects include, for instance, subjects having a genetic abnormality, the presence of which has been demonstrated to have a correlative relation to a higher likelihood of developing a cancer and subjects exposed to cancer causing agents such as tobacco, asbestos, or other chemical toxins. When a subject at risk of developing a cancer is treated with CpG on a regular basis, such as monthly, the subject will maintain a primed set of immune cells which are capable of recognizing and producing an antigen specific immune reponse. If a tumor begins to form in the subject, the subject will develop a specific immune response against one or more of the tumor antigens.

This aspect of the invention is particularly advantageous when the antigen to which the subject will be exposed is unknown. For instance, in soldiers at risk of exposure to biowarfare, it is generally not known what biological weapon to which the soldier might be exposed. A subject traveling to foreign countries may likewise not know what infectious agents they might come into contact with. By inducing immune system remodeling the immune system will be primed to respond to any antigen.

The antigen may be delivered to the immune system of a subject alone or with a

from the simian virus, papilloma virus, adenovirus, human immunodeficiency virus (HIV), rous sarcoma virus, cytomegalovirus, the long terminal repeats (LTR) of moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote transcription and translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined antigen nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

The antigen nucleic acid is operatively linked to the gene expression sequence. As used herein, the antigen nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the expression or transcription and/or translation of the antigen coding sequence under the influence or control of the gene expression sequence. Two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the antigen sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the antigen sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to an antigen nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that antigen nucleic acid sequence such that the resulting transcript is translated into the desired protein or polypeptide.

The antigen nucleic acid of the invention may be delivered to the immune system alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antigen nucleic acid to the cells of the immune system and

advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well-known to those of skill in the art. See e.g., Sanbrook et al., "Molecular Cloning: A Laboratory Manual," Second Edition, Cold Spring Harbor Laboratory Press, 1989. In the last few years, plasmid vectors have been found to be particularly advantageous for delivering genes to cells *in vivo* because of their inability to replicate within and integrate into a host genome. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well-known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA.

It has recently been discovered that gene carrying plasmids can be delivered to the immune system using bacteria. Modified forms of bacteria such as *Salmonella* can be transfected with the plasmid and used as delivery vehicles. The bacterial delivery vehicles can be administered to a host subject orally or by other administration means. The bacteria deliver the plasmid to immune cells, e.g. dendritic cells, probably by passing through the gut barrier. High levels of immune protection have been established using this methodology.

The CpG oligonucleotides of the invention are immune remodeling nucleic acid molecules. An "immune remodeling nucleic acid molecule" refers to a nucleic acid molecule, which contains an unmethylated cytosine-guanine dinucleotide sequence (i.e. "CpG DNA" or DNA containing a 5' cytosine followed by 3' guanosine and linked by a phosphate bond) and stimulates the repopulation of immune cells. An immune remodeling nucleic acid molecule can be double-stranded or single-stranded. Generally, double-stranded molecules are more

sufficient immunostimulatory motifs are present, since larger nucleic acids are degraded into oligonucleotides inside of cells. Preferred synthetic oligonucleotides do not include a CCGG quadmer or more than one CCG or CGG trimer at or near the 5′ and/or 3′ terminals. Stabilized oligonucleotides, where the oligonucleotide incorporates a phosphate backbone modification, as discussed in more detail below are also preferred. The modification may be, for example, a phosphorothioate or phosphorodithioate modification. Preferably, the phosphate backbone modification occurs at the 5′ end of the nucleic acid for example, at the first two nucleotides of the 5′ end of the oligonucleotide. Further, the phosphate backbone modification may occur at the 3′ end of the nucleic acid for example, at the last five nucleotides of the 3′ end of the nucleic acid. Alternatively the oligonucleotide may be completely or partially modified.

Preferably the CpG Oligonucleotide is in the range of between 8 and 100 and more preferably between 8 and 30 nucleotides in size. Alternatively, CpG oligonucleotides can be produced on a large scale in plasmids, which after being administered to a subject are degraded into oligonucleotides.

The CpG oligonucleotide may be directly administered to the subject or it may be administered in conjunction with a nucleic acid delivery complex. A "nucleic acid delivery complex" shall mean a nucleic acid molecule associated with (e.g. ionically or covalently bound to; or encapsulated within) a targeting means (e.g. a molecule that results in higher affinity binding to target cell (e.g. dendritic cell surfaces and/or increased cellular uptake by target cells). Examples of nucleic acid delivery complexes include nucleic acids associated with: a sterol (e.g. cholesterol), a lipid (e.g. a cationic lipid, virosome or liposome), or a target cell specific binding agent (e.g. a ligand recognized by target cell specific receptor). Preferred complexes should be sufficiently stable *in vivo* to prevent significant uncoupling prior to internalization by the target cell. However, the complex should be cleavable under appropriate conditions within the cell so that the nucleic acid is released in a functional form.

"Palindromic sequence" shall mean an inverted repeat (i.e. a sequence such as ABCDEE′D′C′B′A′ in which A and A′ are bases capable of forming the usual Watson-Crick base pairs. *In vivo*, such sequences may form double-stranded structures. In one embodiment the CpG oligonucleotide contains a palindromic sequence. A palindromic sequence used in this context refers to a palindrome in which the CpG is part of the palindrome, and preferably is the center of the palindrome. In another embodiment the CpG oligonucleotide is free of a

Other stabilized oligonucleotides include: nonionic DNA analogs, such as alkyl- and aryl-phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

The nucleic acid sequences of the invention which are useful for inducing immune remodeling are those broadly described above. Exemplary sequences include but are not limited to those sequences shown in Table 1 as well as TCCATGTCGCTCCTGATGCT (SEQ ID NO: 47), TCCATGTCGTTCCTGATGCT (SEQ ID NO: 48), TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 53), TCGTCGTTGTCGTTGTCGTT (SEQ ID NO: 89); TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO: 90), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO: 91), GCGTGCGTTGTCGTTGTCGTT (SEQ ID NO: 92), TGTCGTTTGTCGTTTGTCGTT (SEQ ID NO: 94), TGTCGTTGTCGTTGTCGTT (SEQ ID NO: 96) TCGTCGTCGTCGTT (SEQ ID NO:97), TCCTGTCGTTCCTTGTCGTT (SEQ ID NO: 79), TCCTGTCGTTTTTTGTCGTT (SEQ ID NO:81), TCGTCGCTGTCTGCCCTTCTT (SEQ ID NO:82), TCGTCGCTGTTGTCGTTTCTT (SEQ ID NO:83), TCGTCGTTTTGTCGTTTTGTCGTT (SEQ ID NO:90), TCGTCGTTGTCGTTTTGTCGTT (SEQ ID NO:91) TGTCGTTGTCGTTGTCGTT (SEQ ID NO:96), TCCATGACGTTCCTGACGTT (SEQ ID NO:100), GTCG(T/C)T (SEQ ID NO:101) and TGTCG(T/C)T (SEQ ID NO:102).

The ability of a particular CpG oligonucleotide to induce immune system remodeling can be tested in various immune cell assays which assess the stimulation index of the oligonucleotide. Preferably, the stimulation index of the CpG oligonucleotide with regard to B cell proliferation is at least about 5, preferably at least about 10, more preferably at least about 15 and most preferably at least about 20 as determined by incorporation of ^{3}H uridine in a murine B cell culture, which has been contacted with 20 μM of ODN for 20h at 37°C and has been pulsed with 1 μCi of ^{3}H uridine; and harvested and counted 4h later as described in detail in copending PCT Published Patent Applications claiming priority to U.S. Serial Nos. 08/738,652 and 08/960,774, filed on October 30, 1996 and October 30, 1997 respectively. For use *in vivo*, for example to induce immune system remodeling, it is important that the CpG oligonucleotide be capable of effectively inducing production of APC's such as dendritic

The term "effective amount" of a CpG oligonucleotide refers to the amount necessary or sufficient to realize a desired biologic effect. For example, an effective amount of an oligonucleotide containing at least one unmethylated CpG for treating an immune system deficiency could be that amount necessary to cause repopulation of the immune system, resulting in the development of an antigen specific immune response upon exposure to antigen. The effective amount for any particular application can vary depending on such factors as the disease or condition being treated, the particular CpG oligonucleotide being administered (e.g. the number of unmethylated CpG motifs or their location in the nucleic acid), the size of the subject, or the severity of the disease or condition. One of ordinary skill in the art can empirically determine the effective amount of a particular oligonucleotide without necessitating undue experimentation.

In addition to inducing immune system remodeling by regulating hematopoiesis, the invention relates to methods inducing hematopoiesis of specific immune cells such as platelets and erythroblasts. Such methods are useful for treating thrombocytopenia and anemia respectively.

Thrombocytopenia is a disorder associated with a deficiency in platelets. Platelets, which play an important role in blood coagulation, are derived by cytoplasmic fragmentation of the precursor stem cells, megakaryocytes, found in bone marrow. After formation, platelets leave the bone marrow and travel through the spleen and into the blood, with approximately one third of the platelets becoming sequestered in the spleen. The platelets which are transported to the blood, circulate for approximately seven to ten days. Platelets which are normally present in human blood at a concentration of 150,000-400,000 per microliter play a crucial role in hemostasis, or the regulation of bleeding. When the level of platelets falls below normal in a subject, the risk of hemorrhage increases in the subject.

Ordinarily when the level of circulating platelets decreases a feedback mechanism is initiated which results in increased production in the number, size, and ploidy of megakaryocytes. This mechanism, in turn, causes the production and release into the circulation of additional platelets. Although the feed back regulation of platelet levels is ordinarily sufficient to maintain a normal level of platelets in the circulation, several physiological conditions are capable of causing a significant imbalance in the level of platelets. Such conditions result in either thrombocytopenia or thrombocytosis (a condition caused by an increased level of platelets in the blood).

these therapeutic modalities provides only temporary relief and is associated with serious side effects. Additionally, approximately 20 percent of the chronic ITP patients do not respond to any of the known treatments.

The present invention is a method of treating thrombocytopenia in a subject exhibiting thrombocytopenia, or at risk of developing thrombocytopenia. As used herein, "thrombocytopenia" is a disorder in which the platelet levels in the affected individual fall below a normal range of platelets for that individual.

Thrombocytopenia includes infection-induced thrombocytopenia, treatment-induced thrombocytopenia, and physiologically-induced thrombocytopenia. Infection-induced thrombocytopenia is a disorder characterized by a low level of platelets in peripheral blood which is caused by an infectious agent such as a bacteria or virus. Treatment-induced thrombocytopenia is a disorder characterized by a low level of platelets in peripheral blood which is caused by therapeutic treatments such as gamma irradiation, therapeutic exposure to radiation, cytotoxic drugs, chemicals containing benzene or anthracene and even some commonly used drugs such as chloramphenicol, thiouracil, and barbiturate hypnotics. Physiologically-induced thrombocytopenia is a disorder characterized by a low level of platelets in peripheral blood which is caused by any mechanism other than infectious agents or therapeutic treatments causing thrombocytopenia. Factors causing physiologically-induced thrombocytopenia include, but are not limited to, rare bone marrow disorders such as congenital amegakaryocytic hypoplasia and thrombocytopenia with absent radii (TAR syndrome), an increase in spleen size, or splenomegaly, caused by portal hypertension secondary to liver disease, or macrophage storage disorders such as Gauchers disease, autoimmune disorders such as idiopathic thrombocytopenic purpura (ITP), vasculitis, hemolytic uremic syndrome, thrombotic thrombocytopenic purpura (TTP) disseminated intravascular coagulation (DIC) and prosthetic cardiac valves.

A subject having thrombocytopenia is a subject having any type of thrombocytopenia. In some embodiments the subject having thrombocytopenia is a subject having non-chemotherapeutic induced thrombocytopenia. A subject having non-chemotherapeutic thrombocytopenia is a subject having any type of thrombocytopenia but who is not undergoing chemotherapy. In other embodiments the subject is a subject having chemotherapeutic induced thrombocytopenia, which includes any subject having thrombocytopenia and being treated with chemotherapeutic agents.

measured by any conventional method known in the art for measuring platelet levels or for measuring parameters which correlate with platelet levels. Platelet count is determined simply by obtaining a blood sample and counting the number of platelets per microliter of blood. Platelet levels also can correlate with bleeding time.

The invention is particularly useful for the early treatment of thrombocytopenia after a thrombocytopenic triggering event. As shown in the examples below, when a subject exposed to a thrombolytic triggering event is administered a CpG oligonucleotide the subject has an increased platelet count compared to a subject exposed to the thrombocytopenia triggering event but not treated with a CpG oligonucleotide. The response is particularly significant in a short period of time after the subject is exposed to the triggering event. For example, a significant increase in platelet counts is observed after four days.

Anemia is a blood disorder associated with a decrease in levels of red blood cells or erythrocytes. Erythrocytes are derived from the same undifferentiated progenitor cell in the bone marrow as platelets, referred to as the pluripotent stem cell. The pluripotent stem cell can generate an erythroid burst forming unit which can in turn form an erythroid colony forming unit. These cells eventually differentiate into erythroblasts, followed by erythrocytes.

In one aspect the invention is a method for treating anemia by administering to a subject having anemia an oligonucleotide, having a sequence including at least the following formula:

$$5' X_1CGX_2 3'$$

wherein the oligonucleotide includes at least 8 nucleotides wherein C and G are unmethylated and wherein X_1 and X_2 are nucleotides, in an amount effective to induce erythropoiesis in the subject.

The amount of erythroblasts in a subject can be assessed by measuring the number of erythroblasts in bone marrow or by measuring the amount of erythrocytes in peripheral blood. The assay involving the measurement of erythrocytes in peripheral blood is more convenient and provides reasonable correlation to the number of erythroblasts.

"Anemia" as used herein refers to a disease in which there is a loss in number of red blood cells and/ or hemoglobin concentration. An anemic subject usually experiences a reduction in blood cell mass and a corresponding decrease in the oxygen carrying capacity of the blood. Many types of underlying disease cause anemia. These are discussed in extensive

Table below.

Normal Hematology Values for Dogs and Cats		
Unit	Canine	Feline
Hematocrit (PCV) %	40-59	29-50
Hemoglobin g/dl	14-20	9-15.6
Red BloodCell Count x106/ml	5.6-8.7	6.1-11.9
White Blood Cell Count/m l	6,000-17,000	4,900-20,000
Neutrophils/m l	3,000-12,000	2,500-12,500
Lymphocytes/m l	530-4,800	1,500-7,000
Monocytes /m l	100-1800	0-850
Eosinophils/m l	0-1,900	0-1,500
Basophils /m l	<100	<100
Platelets/m l	145-440	190-800

Horses also develop hematopoietic disorders such as anemia. One anemic condition that horses develop is an exercise induced increase in the number of crenated or spiculated red blood cells as described in US Patent No. 4,500,530. The red blood cell spiculation results in destruction of the cells leading to sports anemia. The methods of the invention may be used to treat or prevent this disorder in animals undergoing exercise. For instance, horses may be administered CpG prior to or after a race to prevent or treat anemia.

The CpG oligonucleotide useful according to the methods of the invention is the CpG oligonucleotide described above. The preparations of the invention are administered in effective amounts. An effective amount of an oligonucleotide is that amount that will alone, or together with further doses, desirably modulate platelet or erythroblast levels such as by increasing the circulating level of platelets or erythroblasts of a subject. It is believed that doses ranging from 1 nanogram/kilogram to 100 milligrams/kilogram, depending upon the mode of administration, will be effective. The preferred range is believed to be between 0.1 and 10.0 mg/dose, particularly if given subcutaneously. More preferably, the amount is in the

chlorobutanol (0.3-0.9% w/v); parabens (0.01-0.25% w/v) and thimerosal (0.004-0.02% w/v).

The pharmaceutical compositions of the invention contain an effective amount of a CpG oligonucleotide and antigens optionally included in a pharmaceutically-acceptable carrier. The term "pharmaceutically-acceptable carrier" means one or more compatible solid or liquid filler, dilutants or encapsulating substances which are suitable for administration to a human or other vertebrate animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being commingled with the compounds of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficiency.

Compositions suitable for parenteral administration conveniently comprise sterile aqueous preparations, which can be isotonic with the blood of the recipient. Among the acceptable vehicles and solvents are water, Ringer's solution, and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid find use in the preparation of injectables. Carrier formulations suitable for subcutaneous, intramuscular, intraperitoneal, intravenous, etc. administrations may be found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, PA.

The CpG oligonucleotides or antigens useful in the invention may be delivered in mixtures of more than one CpG oligonucleotide or antigen. A mixture may consist of several CpG oligonucleotides or antigens.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular CpG oligonucleotide or antigen selected, the particular condition being treated and the dosage required for therapeutic efficacy. The methods of this invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of an immune response without causing clinically unacceptable adverse effects. Preferred modes of administration are discussed above.

Examples:

Example 1: CpG Oligonucleotides Induce Hematopoiesis.

Methods

Mice. Female C57BL/6, BALB/c, CBA/J, C3H/HeJ and SCID mice were purchased from Harlan Winkelmann (Borchen, Germany), Charles River Wiga (Sulzfeld, Germany) or Bomholtgard Breeding and Research Centre Ltd. (Ry, Denmark). All animals were housed in specific pathogen-free conditions and were used at 8-12 weeks of age (18 to 21 g of body weight).

Tissues and cells. Femurs and spleens were aseptically removed and collected into ice-cold mouse tonicity PBS. Single cell suspensions were prepared and clumps were removed using a 100 µm pore size filter (Falcon, Becton Dickinson, Heidelberg, Germany). For the depletion of B (B220 positive) and T cells (CD4 or CD8 positive) cells, spleen cells were incubated with magnetic beads coated with the respective antibodies allowing negative selection of the splenic non B and non T cell portion (Dynal, Hamburg, Germany). Efficiency was checked by FACS-analysis, yielding in <5% B220 and <3% CD3 positive cells after depletion.

Microbial stimuli and synthetic oligonucleotides. Phosphorothioate-stabilized oligonucleotides (ODN) were synthesized by TibMolBiol (Berlin, Germany). ODN sequences 'CG1' (= ODN 1668, containing a 'CG-motif' marked with bold letters: 5'-TCC-AT**G-ACG-TT**C-CTG-ATG-CT) (SEQ ID NO. 24) and control GC-ODN ('inverted CG' = ODN 1720: 5'-TCC-AT**G-AGC-TT**C-CTG-ATG-CT)(SEQ Id NO. 106) were taken from Krieg, AM et al. (1995) Nature 374:546-549. A second CpG-ODN 'CG2' (= ODN IL12p40: 5'-AGC-TAT-**GAC-GTT**-CCA-AGG) (SEQ ID NO. 107) and control ODN 'nCG' ('non-CG' = ODN AP1, without CG-motif: 5'-GCT-TGA-TGA-CTC-AGC-CGG-AA) (SEQ ID NO. 108) were described recently. Lipford, GB et al. (1997) Eur J Immunol 27:2340-2344. LPS from *E. coli* was purchased from Sigma (Munich, Germany). *Listeria monocytogenes* came from ATCC (American type culture collection strain 43251) and were grown in brain hear infusion (Difco, Detroit, USA) in overnight cultures. Number of bacteria was determined by OD_{600} and checked by plating 10 µl aliquots of a serial 10-fold dilution on Columbia blood agar plates and counting the colony forming units after overnight incubation at 37°C.

Treatment of mice. CpG-ODN were injected intraperitoneally (i.p.) in low endotoxin

for GM-CFU by a soft agar colony assay as described previously. Staber, FG et al. (1982) Nature 298:79-82. In brief, the desired number of spleen cells (final concentration usually $3x10^5$ to $1x10^6$ per ml) was added to the agar medium mixture and 1 ml was added in triplicate to 35-mm-diameter culture plates (Greiner, Nürtingen, Germany). Prior to cell plating a saturating amount of a pre-tested cocktail of myeloid cell growth promoting cytokines including mu r hisKL, mu r IL-3 and r GM-CSF (50 µl/plate, respectively) had been added to the plates corresponding to final concentrations of 500 ng/ml hisKL, 5 ng/ml IL-3, and 25 ng/ml GM-CSF. After gelling of the agar medium at 4°C the cultures were incubated for 7 days at 37°C in a fully humidified atmosphere of 10% CO_2 in air. Cellular aggregates containing at least 50 cells were scored as colonies.

BFU-E Assays. A commercially available (CellSystems Biotechnologie Vertrieb GmgH, Remagen, Germany) culture medium composition (MethoCult™ HCC-3340) was used which contained 0.9% methylcellulose in alpha modified Eagle's medium. 30% foetal bovine serum 1% BSA, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine and 3 units/ml r human (hu) erythropoietin. To this medium (2.7 ml/tube) 0.3 ml cell suspension was added containing $13.2x10^5$/ml spleen cells. The culture medium was further complemented with 100 µl mu r hisKL (stock: 10 µg/ml), 100 µl mu r IL-3 (stock: 1 µg/ml), and 100 µl hu r IL-6 (stock: 100 ng/ml) and carefully mixed with a 1.4x40m needle. This resulted in a final concentration of 3 µg/ml hisKL, 3 ng/ml mu r IL-3, 3 ng/ml hu r IL-6 and $4x10^5$/ml spleen cells which were plated in triplicate aliquots of 1 ml per Petri dish (Greiner, Nürtingen, Germany). Growth of erythroid colonies (< 50 hemoglobin containing cells) was scored after an incubation period of 9 days at 37°C in a humidified atmosphere containing 10% CO_2 in air.

Day-11 CFU-Assay. Spleen colony forming units (CFU-S) were measured by the macroscopic spleen colony assay of Till and McCulloch. Female C57BL/6 mice at the age of 12 weeks were irradiated with 8 Gy (^{137}Cs), a potentially lethal dose which was found to give no formation of endogenous macroscopic spleen colonies. Within a period of 1 to 4 hours, the irradiated mice were anaesthetized with diethylether and injected into the retro-orbital plexus with $2.5x10^5$ spleen cells/200 µl/mouse derived from individual normal C57BL/6 mice or from mice sacrificed 6 days after i.p. treatment with 10 nmol/mouse CpG-ODN (5 mice group treated with CpG-ODN or vehicle, respectively). Each donor spleen suspension was injected

dramatic effect observed however was a transient but significant increase at day 6 in the B220-CD3 double negative compartment. Histologically, an increased number of large immature blasts and erythroblasts was detected with a maximum at day 6 suggesting increased hematopoietic activity.

Fig. 2 shows changes in phenotype of spleen cells after stimulation with CpG-ODN. CpG-ODN (CG1) was injected once i.p. at day 0 (10 nmol/mouse). Spleens were removed at indicated time points and FACS-stained for B220/CD3 and GR-1/Mac-1 (double stainings). Increase of absolute cell number is presented as factor over day 0 control spleen cells (mean values of 3 individual C57BL/6 mice).

Splenomegaly is associated with extramedullary hematopoiesis. In contrast to humans, mice display a basal hematopoietic activity in the spleen. Morrison, SJ et al. (1995) Annu Rev Cell Dev Biol 11:35-71. To analyze whether CpG-ODN induced splenomegaly correlated with increased splenic hematopoietic activity, we measured the number of granulocyte-macrophage progenitor cells (GM-CFU) in spleens of CpG ODN treated mice. There was a 7.4-fold increase in splenic GM-CFU numbers at day 6, reflecting the kinetics of total spleen cell number (Fig. 3A, 3B). We also analyzed the induction of GM-CFU in bone marrow from treated mice. There was a slight increase in the number of GM-CFU in bone marrow (day 4) that preceded the splenic increase at day 6, as if mobilization of bone-marrow derived progenitor cells to the spleen may have taken place (Fig. 3C). In addition, we enriched by immunomagnetic separation the B220/CD3 double negative cell fraction from day 6 spleens of CpG or non-CpG treated mice and tested for GM-CFU formation. These cells were shown to be highly enriched for myeloid progenitor cells (Fig. 3D). Thus the dramatic increase of the non-B, non-T cell fraction at day 6 post CpG-ODN injection was accompanied by an increased number of GM-CFU within the spleen.

Fig. 3 shows CpG-ODN induced changes in splenic cell number, number of splenic and BM GM-CFU. A: Kinetics of CpG-ODN (CG1) induced changes in splenic cell count (mean values of 3 C57BL/6 mice per time point ± SD). B: Evaluation of hematopoietic progenitor cells in the spleens of CpG-ODN-treated mice. Graph display number of GM-CFU per spleen per time point (mean values of triplicate spleen cell cultures of 3 mice ± SEM). C: Frequency of GM-CFU in pooled bone-marrow cells from 3 mice per time point. D: Increased number of GM-CFU in B220/CD3 double negative spleen cell fraction. Spleen

characteristics of primitive hematopoietic stem cells such as extensive proliferative capacity, the ability for self-renewal and the capability of generating spleen colonies containing cells of multiple hematopoietic lineages that can rescue animals from lethal irradiation. Spangrude, GJ et al. (1988) Science 241:58-62. In view of this data experiments were designed to examine the reconstitution of lethally irradiated mice by adoptive transfer of CFU-S contained in spleens of CpG-ODN treated mice.

Fig. 6 shows a determination of spleen colony forming units of normal vs. CpG-ODN induced spleen cells (CFU-S Assay). CpG-ODN (CG1) induced splenic hematopoiesis leads to increased number of macroscopic visible colonies after injection into lethally irradiated mice. Graph displays numbers of macroscopic nodules per spleen of untreated mice after lethal irradiation (grey bar) compared to lethally irradiated mice after injection of $2.5x10^5$ normal spleen cells (white bar) and irradiated mice injected with spleen cells from ODN-pre-treated mice (day 6 post ODN CG1, black bar) (mean values of 5 independent experiments using 3-5 C57BL/6 mice per spleen ± SEM).

CpG-ODN mediate radioprotective effects in myelosuppression. Hematopoietic progenitor cells are considered as rather radioresistant. Morrison, SJ et al. (1995) Annu Rev Cell Dev Biol 11:35-71. Since CpG-DON induce extramedullary hematopoiesis via mobilization of CFU-S to the spleen we analyzed whether CpG-ODN could mediate radioprotective effects in sublethally irradiated mice. CpG challenge of sublethally irradiated mice (4 Gy) lead within day s to a 4 fold increase lead within 14 days to a 4 fold increase of splenic GM-CFU (Fig. 7A). Next, we addressed the question whether CpG-ODN driven hematopoiesis in sublethally irradiated mice allows accelerated recovery of the immune system. Two experimental systems were chosen: one, the induction of CTL responses to proteinaceous antigens (Lipford, GB et al. (1997) Eur J Immunol 27:2340-2344), and two, resistance to the intracellular pathogen *Listeria monocytogenes* (Endres, R et al. (1997) Immunity 7:419-432). Mice were treated with CpG-ODN within 30 minutes after sublethal irradiation (4 Gy), allowed to recover for 18 days and thereafter immunized subcutaneously (s.c.) with ovalbumin (OVA) containing liposomes plus QuilA as adjuvant. After 4 days cells of draining lymph nodes were harvested, cultured for an additional four days and assayed for OVA specific CTL activity. As detailed in Fig. 7B lymphocytes from CpG-ODN treated irradiated mice displayed an enhanced CTL response compared to non-treated irradiated mice.

157:2116-2122; Lipford, GB et al. (1997) Eur J Immunol 27:3420-3426. CpG-ODN activate DC and macrophages in vitro to secrete large amounts of hematopoietically active cytokines including IL-6, GM-CSF, IL-1, 1L-2 and TNF-α. Sparwasser, T et al. (1997) Nature 386:336-337; Sparwasser, T et al. (1997) Eur J Immunol 27:1671-1679; Sparwasser, T et al. (1998) Eur J Immunol 28:2045-2054; Lipford, GB et al. (1997) Eur J Immunol 27:3420-3426; Halpern, MD et al. (1996) Cell Immunol 167:72-78; Chace, JH et al. (1997) Clin Immunol Immunopathol 84:185-193; Roman, M et al. (1997) Nat Med 3:849-854 31-33. Mice challenged with CpG-ODN also transiently exhibit high serum concentrations of these cytokines. Sparwasser, T et al. (1997) Nature 386:336-337; Lipford, GB et al. (1997) Eur J Immunol 27:3420-3426. To date it is unclear which of these triggers extramedullary hematopoiesis. It is possible that CpG-ODN target bone marrow stroma cells to release hematopoietically active cytokines.

Initially, we anticipated that the observed splenomegaly reflected CpG-ODN induced B cell mitogenicity because most references attribute CpG induced splenomegaly to B cells. Krieg, AM et al. (1995) Nature 374:546-549; McIntyre, KW et al. (1993) Antisense Res Dev 3:309-322; Branda, RF et al. (1993) Biochem Pharmacol 45:2037-2043. However it was only between days 1-4 after CpG-ODN challenge that proliferating $B220^+$ cells account for the relative increase in splenic cellularity (Fig. 2). Supporting a conclusion of non-B, non-T cell involvement in splenomegaly, spleen enlargement was also observed in SCID-mice which lack B and T cells. At day 6 after CpG-ODN challenge $B220^-/CD3^-$ splenic cells were prevalent (Fig. 2), and histology revealed abundant large immature blast cells indicative for extramedullary hematopoiesis. In GM-CFU in vitro assays the increased hematopoietic activity could be defined to the $B220^-/CD3^-$ population. In vitro colony assays (Figs. 4, 5, 6, Table 8) demonstrated massive increase in splenic numbers of granulocyte, macrophage and early erythrocyte progenitor cells. In peripheral blood of the mice however, changes were discrete in that leukocytosis and a slight reduction of numbers of erythrocytes and platelets were observed. Unlike humans, the spleen of mice accounts for a large portion of hematopoietic activity.

It is known that bacterial stimuli (LPS or complete Freud's adjuvant containing heat killed mycobacteria) can trigger increased splenic hematopoiesis (Apte, RN et al. (1976) J Cell Physiol 71-78; Staber, FG et al. (1980) Proc Natl Acad Sci USA 77:4322-4325; McNeill,

treated (10 nmol/mouse) and mock-treated mice (injection with aqua ad injectable). b) Number of GM-CFU per spleen (mean values of triplicate values of 3 C57BL/6 mice per group ± SEM). c) Number of GM-CFU per 1 million cells (mean values of triplicate values of 3 mice per group ± SEM).

Example 2: CpG-ODN Induced Blood and Cell Resistance to 5-Fluorouracil (5-FU).

Two groups of BALB/c mice, 9 mice each at 10 weeks of age, were injected intraperitoneally (i.p.) with 150 mg/kg of 5-FU in 200 µl of sterile phosphate buffered saline (PBS) on day 0. A third group of BALB/c mice, 9 mice at 10 weeks of age, were injected i.p. with 200 µl of sterile PBS alone on day 0. Twenty-four hours later one group of 5-FU treated mice were administered 3 mg/kg CpG-ODN (CG1) in 200 µl sterile PBS; the other 5-FU treated group and the PBS-treated group received PBS alone. This resulted in three experimental groups: mock treatment (Mock), 5-FU treatment (5-FU), and combined treatment with 5-FU plus CpG-ODN (5-FU + ODN). On days 4, 7 and 10 following 5-FU treatment, 3 mice from each group were sacrificed and assays were performed to access immunoresistance to chemotherapeutic treatment.

1. **Spleen Weight and Spleen Cell Count.** Spleens removed on days 0, 4, and 10 were trimmed of fat and contiguous tissues, and then weighed. They then were minced and dispersed for cell counting. Red blood cells were removed by NH_4Cl lysis prior to cell counts. As shown in Fig. 8, spleens from animals treated with 5-FU plus CpG-ODN weighed more on days 4 and 10 following 5-FU treatment than did spleens from animals receiving 5-FU alone, and spleen cell counts tended to be higher and closer to normal in animals receiving combined treatment than in those receiving 5-FU alone.

2. **Differential Splenic Lymphocyte Counts Following 5-FU with and Without CpG-ODN.** Splenic lymphocytes ($5x10^5$ to $1x10^6$) were washed in PBS containing 2% fetal calf serum and incubated for 10 minutes at 4°C with anti-FcγRII/III antibodies to block nonspecific binding of FITC-labeled anti-B220 or anti-CD3. Cells were washed between 30 minute incubation steps with 1:1 PBS/FCS. FACS analysis was performed using a Coulter Epics XL flow cytometer, acquiring 10,000 events per data point. As shown in Fig. 9, T cells were decreased on day 4 in animals treated with 5-FU alone and recovered to normal by day

Mice receiving 5-FU plus CpG-ODN exhibited by comparison a much stronger CTL response than observed in the 5-FU alone group. Thus an effect of the administration of CpG-ODN in conjunction with 5-FU was to preserve the ability to mount an effective CTL response at a level closer to that observed in untreated animals and distinctly higher than that observed in animals treated with 5-FU alone.

Example 3: Hematopoietic Remodeling

1. Dendritic Cells. Two groups of C57BL/6 mice were administered 3 mg/kg CpG-ODN (CG1) in 200 µl sterile PBS or PBS alone on day 0. Seven days later, mice were sacrificed and spleens harvested as in Example 2 for analysis. Spleens so obtained were subjected to an additional treatment with collagenase, yielding higher total numbers of splenocytes per spleen than obtained in Example 2. Splenocytes then were counted and aliquoted; an aliquot from each treatment group was stained with anti-CD11c and anti-CD11b for FACS analysis to quantitate total resident splenic DCs. As shown in the left panel of Fig. 15, the number of CD11c/CD11b double positive spleen cells in the spleens of animals treated with CpG-ODN was expanded 7-fold over control. Aliquots of remaining portions of the splenocytes harvested on day 7 were propagated in culture for an additional 7 days in the presence of growth factors known to favor DC growth. Sparwasser, T et al. (1998) Eur J Immunol 28:2045-2054. Viable cells in culture were then counted and analyzed by FACS as above to determine the population of CD11c/CD11b double positive cells (DCs) remaining in culture. As shown in the right panel of Fig. 15, splenocytes derived from mice treated with CpG-ODN and propagated under these conditions were highly enriched for DCs, while splenocytes derived from mock-injected mice grew out nearly none ($51x10^6$/spleen vs. $0.6x10^6$/spleen, respectively).

2. Effect of Hematopoietic Remodeling on Induction of Antibody to Antigen. Four groups of C57BL/6 mice were injected with 3 mg/kg CpG-ODN (CG1) in 200 µl sterile PBS; a fifth group was injected with PBS alone. Injected mice then were immunized with OVA according to a fixed schedule spanning 21 days, beginning at different times relative to the CpG-ODN or PBS injection. The immunization protocol consisted of injection of 100 µg OVA, followed by a booster injection of OVA 14 days later. After an additional 7 days of rest, serum samples were collected and analyzed by IgG isotype-specific ELISA, using OVA-

Table 1 -sequences

GCTAGACGTTAGCGT	(SEQ ID NO: 1)
GCTAGATGTTAGCGT	(SEQ ID NO: 2)
GCTAGAZGTTAGCGT	(SEQ ID NO: 3)
GCTAGACGTTAGZGT	(SEQ ID NO: 4)
GCATGACGTTGAGCT	(SEQ ID NO: 5)
ATGGAAGGTCCAGCGTTCTC	(SEQ ID NO: 6)
ATCGACTCTCGAGCGTTCTC	(SEQ ID NO: 7)
ATZGACTCTZGAGZGTTCTC	(SEQ ID NO: 8)
ATZGACTCTCGAGCGTTCTC	(SEQ ID NO: 9)
ATCGACTCTCGAGCGTTZTC	(SEQ ID NO: 10)
ATCGACTCTCGAACGTTCTC	(SEQ ID NO: 11)
GAGAACGCTGGACCTTCCAT	(SEQ ID NO: 12)
GAGAACGCTCGACCTTCCAT	(SEQ ID NO: 13)
GAGAACGCTCGACCTTCGAT	(SEQ ID NO: 14)
GAGCAAGCTGGACCTTCCAT	(SEQ ID NO: 15)
GAGCAZGCTGGACCTTCCAT	(SEQ ID NO: 16)
GAGAACGCTGGACZTTCCAT	(SEQ ID NO: 17)
GAGAACGATGGACCTTCCAT	(SEQ ID NO: 18)
GAGAACGCTCCAGCACTGAT	(SEQ ID NO: 19)
CCATGTCGGTCCTGATGCT	(SEQ ID NO: 20)
TCCATGCTGGTCCTGATGCT	(SEQ ID NO: 21)
TCCATGTZGGTCCTGATGCT	(SEQ ID NO: 22)
TCCATGTCGGTZCTGATGCT	(SEQ ID NO: 23)
TCCATGACGTTCCTGATGCT	(SEQ ID NO: 24)
TCCATGTCGGTCCTGACGCA	(SEQ ID NO: 25)
TCAACGTT	(SEQ ID NO: 26)
TCAAGCTT	(SEQ ID NO: 27)
TCAGCGCT	(SEQ ID NO: 28)
TCTTCGAT	(SEQ ID NO: 29)
TCTTCGAA	(SEQ ID NO: 30)
CAACGTT	(SEQ ID NO: 31)
CCAACGTT	(SEQ ID NO: 32)
CAACGTTCT	(SEQ ID NO: 33)
TCAACGTC	(SEQ ID NO: 34)
ATGGACTCTCCAGCGTTCTC	(SEQ ID NO: 35)
ATAGGAGGTCCAACGTTCTC	(SEQ ID NO: 36)
ATCGACTCTCGAGCGTTCTC	(SEQ ID NO: 37)
ATGGAGGCTCCATCGTTCTC	(SEQ ID NO: 38)
ATZGGACTCTZGAGZGTTCTC	(SEQ ID NO: 39)
ATCGACTCTCGAGZGTTCTC	(SEQ ID NO: 40)
GCATGACGTTGAGCT3'	(SEQ ID NO: 41)
TCCATGTCGGTCCTGATGCT	(SEQ ID NO: 42)
TCCATGCCGGTCCTGATGCT	(SEQ ID NO: 43)
TCCATGGCGGTCCTGATGCT	(SEQ ID NO: 44)

TCGTCGTTGTCGTTTTGTCGTT	(SEQ ID NO: 91)
GCGTGCGTTGTCGTTGTCGTT	(SEQ ID NO: 92)
GCGGCGGGCGGCGCGCGCCC	(SEQ ID NO: 93)
TGTCGTTTGTCGTTTGTCGTT	(SEQ ID NO: 94)
TGTCGTTGTCGTTGTCGTTGTCGTT	(SEQ ID NO: 95)
TGTCGTTGTCGTTGTCGTT	(SEQ ID NO: 96)
TCGTCGTCGTCGTT	(SEQ ID NO: 97)
TGTCGTTGTCGTT	(SEQ ID NO: 98)
TCCATAGCGTTCCTAGCGTT	(SEQ ID NO: 99)
TCCATGACGTTCCTGACGTT	(SEQ ID NO: 100)
GTCG(T/C)T	(SEQ ID NO: 101)
TGTCG(T/C)T	(SEQ ID NO: 102)
TCCATGAGCTTCCTGAGTCT	(SEQ ID NO: 103)
TCTCCCAGCGTGCGCCAT	(SEQ ID NO: 104)
TCCATGACGTTCCTGACGTT	(SEQ ID NO: 105)
AGCTATGACGTTCCAAGG	(SEQ ID NO. 107)

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by examples provided, since the examples are intended as a single illustration of one aspect of the invention and other functionally equivalent embodiments are within the scope of the invention. Various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims. The advantages and objects of the invention are not necessarily encompassed by each embodiment of the invention.

All references, patents and patent publications that are recited in this application are incorporated in their entirety herein by reference.

We claim:

end of the oligonucleotide.

10. The method of claim 1, wherein the oligonucleotide has a sequence including at least the following formula:

5' $X_1X_2CGX_3X_4$ 3'

wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or TpC.

11. The method of claim 1, wherein the oligonucleotide has a sequence including at least the following formula:

5' $TCNTX_1X_2CGX_3X_4$ 3'

wherein X_1, X_2, X_3, and X_4 are nucleotides, N is a nucleic acid sequence composed of from about 0-25 nucleotides.

12. The method of claim 1, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or TpC.

13. The method of claim 1, wherein the antigen is a nucleic acid encoding an antigen.

14. The method of claim 1, wherein the antigen is selected from the group consisting of cells, cell extracts, proteins, polysaccharides, polysaccharide conjugates, lipids, glycolipids, carbohydrate, viral extracts, viruses, bacteria, fungi, parasites, and allergens.

15. The method of claim 1, wherein the antigen is an allergen.

16. The method of claim 1, wherein the antigen is derived from an infectious organism selected from the group consisting of infectious bacteria, infectious viruses, and infectious fungi.

17. The method of claim 1, wherein the subject is actively exposed to the antigen.

wherein the oligonucleotide includes at least 8 nucleotides wherein C and G are unmethylated and wherein X_1 and X_2 are nucleotides, in an amount effective to increase platelet counts in the subject.

28. The method of claim 27 wherein the oligonucleotide is administered in an amount effective to increase platelet counts in the subject by at least 10,000 platelets per microliter.

29. The method of claim 27 wherein the oligonucleotide is administered in an amount effective to increase platelet counts in the subject by at least 20,000 platelets per microliter.

30. The method of claim 27 wherein the oligonucleotide is administered to the subject in an amount effective to increase the platelet counts in the subject by 100 percent.

31. The method of claim 27 wherein the thrombocytopenia is a drug-induced thrombocytopenia.

32. The method of claim 27 wherein the thrombocytopenia is due to an autoimmune disorder such as idiopathic thrombocytopenic purpura.

33. The method of claim 27 wherein the thrombocytopenia is a thrombocytopenia resulting from accidental radiation exposure.

34. The method of claim 27 wherein the thrombocytopenia is a thrombocytopenia resulting from therapeutic radiation exposure.

35. The method of claim 27, wherein the oligonucleotide is 8 to 100 nucleotides in length.

36. The method of claim 27, wherein the oligonucleotide includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

43. The method of claim 42 wherein the subject at risk of developing thrombocytopenia has a disorder treated with platelet suppressive drugs.

44. The method of claim 42, wherein the oligonucleotide is 8 to 100 nucleotides in length.

45. The method of claim 42, wherein the oligonucleotide includes a phosphate backbone modification which is a phosphorothioate or phosphorodithioate modification.

46. The method of claim 45, wherein the phosphate backbone modification occurs at the 5' end of the oligonucleotide.

47. The method of claim 45, wherein the phosphate backbone modification occurs at the 3' end of the oligonucleotide.

48. The method of claim 42, wherein the oligonucleotide has a sequence including at least the following formula:

5' $X_1X_2CGX_3X_4$ 3'

wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or TpC.

49. The method of claim 42, wherein the oligonucleotide has a sequence including at least the following formula:

5' $TCNTX_1X_2CGX_3X_4$ 3'

wherein X_1, X_2, X_3, and X_4 are nucleotides, N is a nucleic acid sequence composed of from about 0-25 nucleotides.

50. The method of claim 42, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or TpC.

59. The method of claim 58, wherein the phosphate backbone modification occurs at the 5' end of the oligonucleotide.

60. The method of claim 58, wherein the phosphate backbone modification occurs at the 3' end of the oligonucleotide.

61. The method of claim 51, wherein the oligonucleotide has a sequence including at least the following formula:

5' $X_1X_2CGX_3X_4$ 3'

wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA; and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or TpC.

62. The method of claim 51, wherein the oligonucleotide has a sequence including at least the following formula:

5' $TCNTX_1X_2CGX_3X_4$ 3'

wherein X_1, X_2, X_3, and X_4 are nucleotides, N is a nucleic acid sequence composed of from about 0-25 nucleotides.

63. The method of claim 51, wherein X_1X_2 are nucleotides selected from the group consisting of: GpT, GpG, GpA and ApA and X_3X_4 are nucleotides selected from the group consisting of: TpT, CpT or TpC.

64. The method of claim 51 wherein the anemia is an anemia resulting from accidental radiation exposure.

65. The method of claim 51 wherein the anemia is an anemia resulting from therapeutic radiation exposure.

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the pamphlet!

WO 99-58118

2/2

Date: 18 nov 1999

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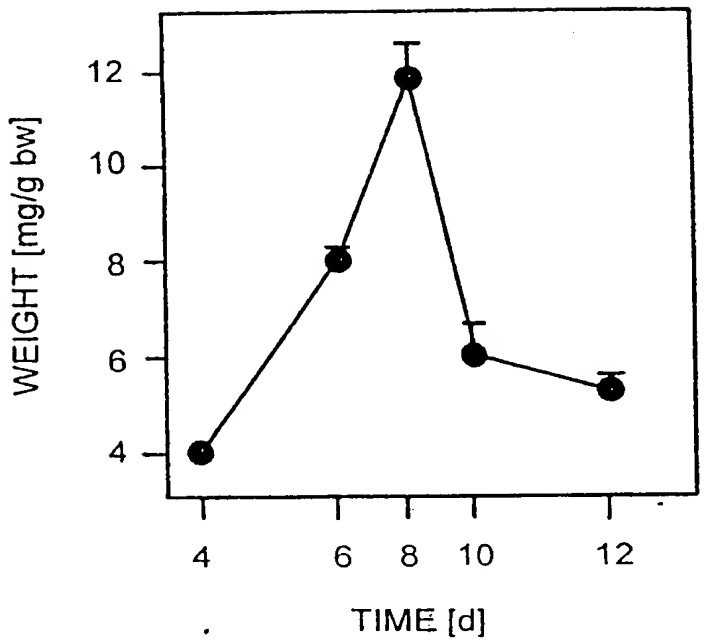
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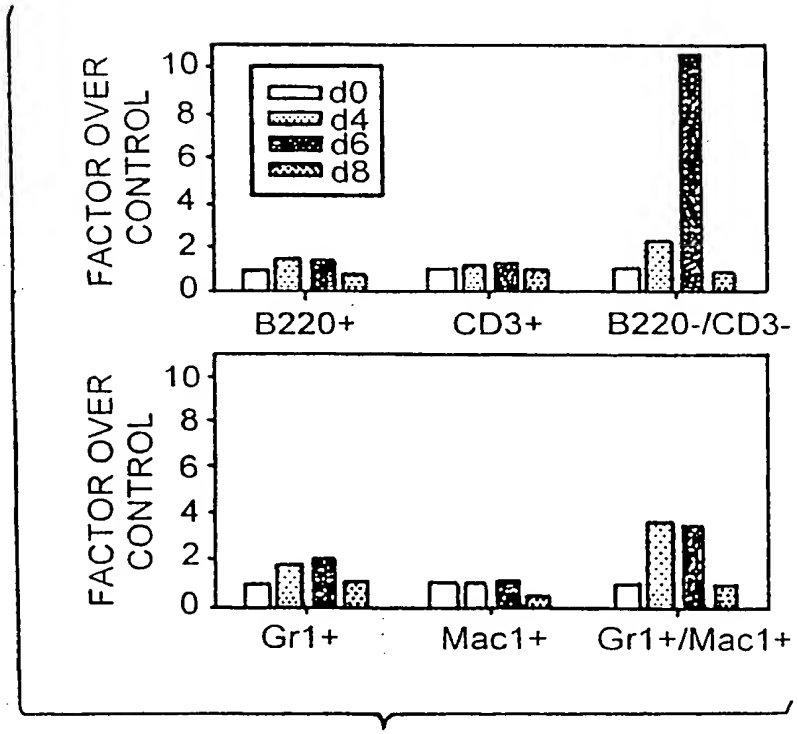
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6
4
2
0
d0
d4
d6
d8
B220+
CD3+
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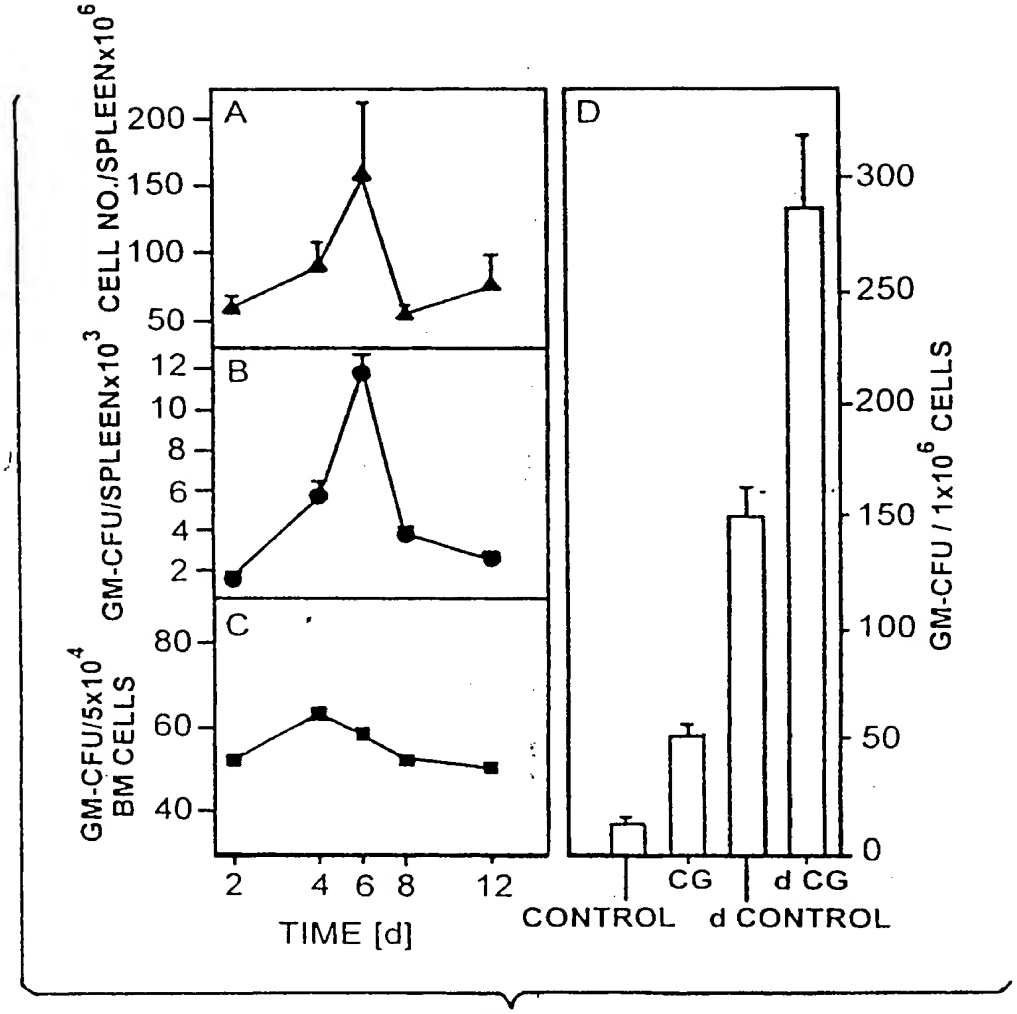
4
2
0
Gr1+
Mac1+
Gr1+/Mac1+

Fig. 2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B
C
D
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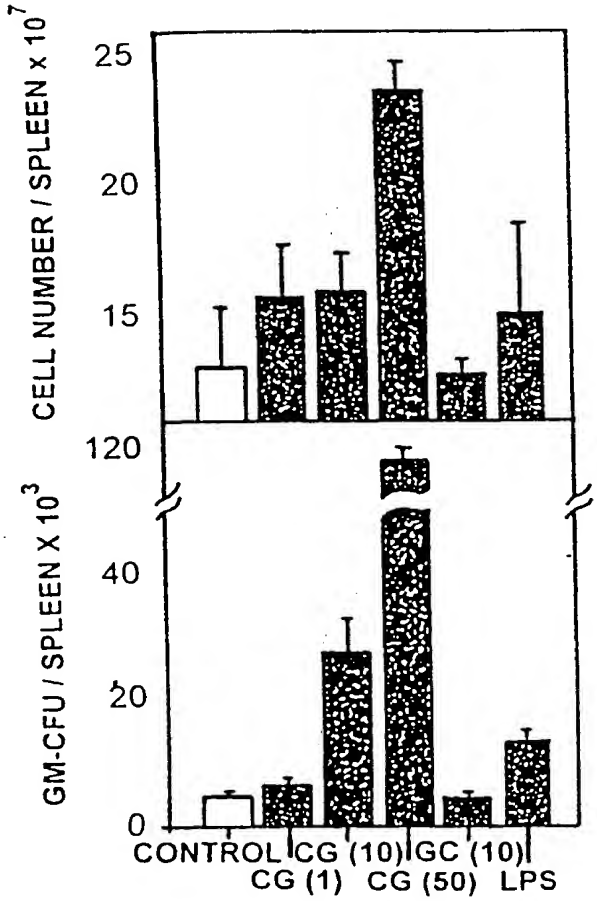
TIME [d]
CONTROL
CG
d CONTROL
d CG

Fig. 3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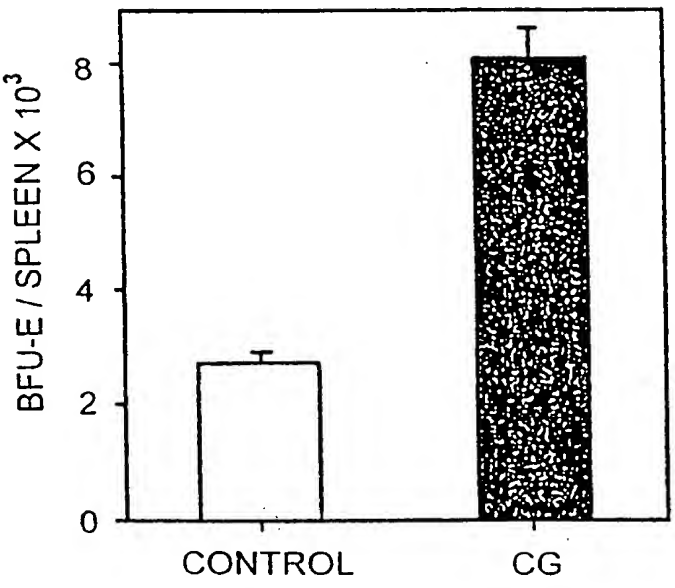
CG (50)
GC (10)
LPS

Fig. 4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4
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CONTROL
CG

Fig. 5

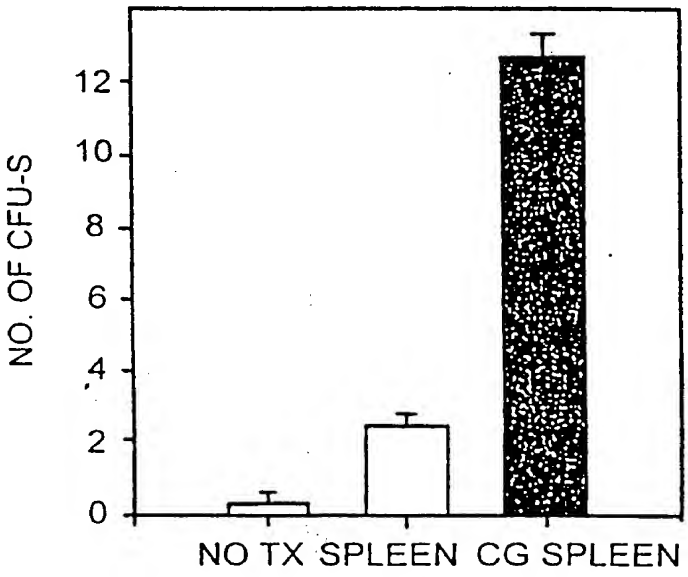


Fig. 6

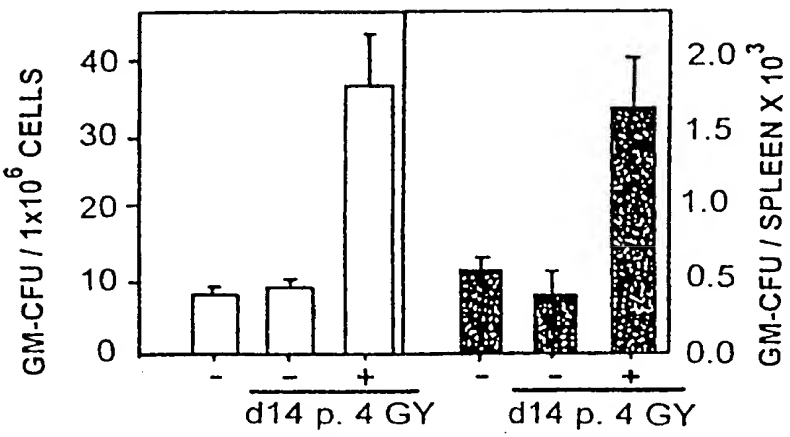


Fig. 7A

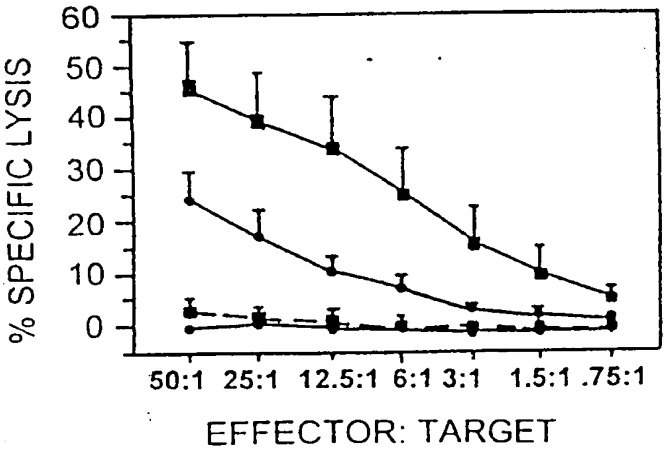


Fig. 7B

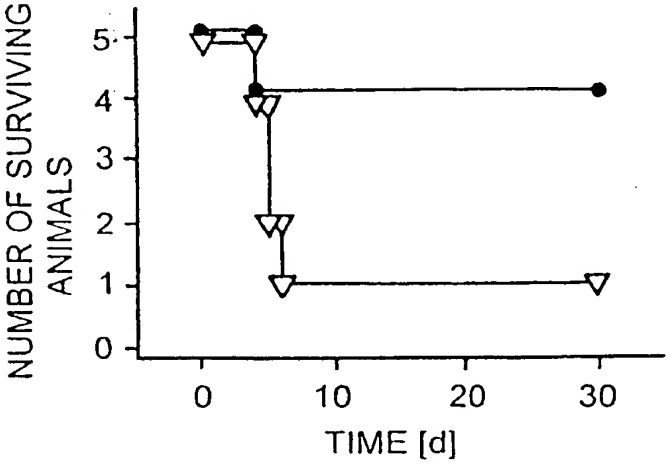
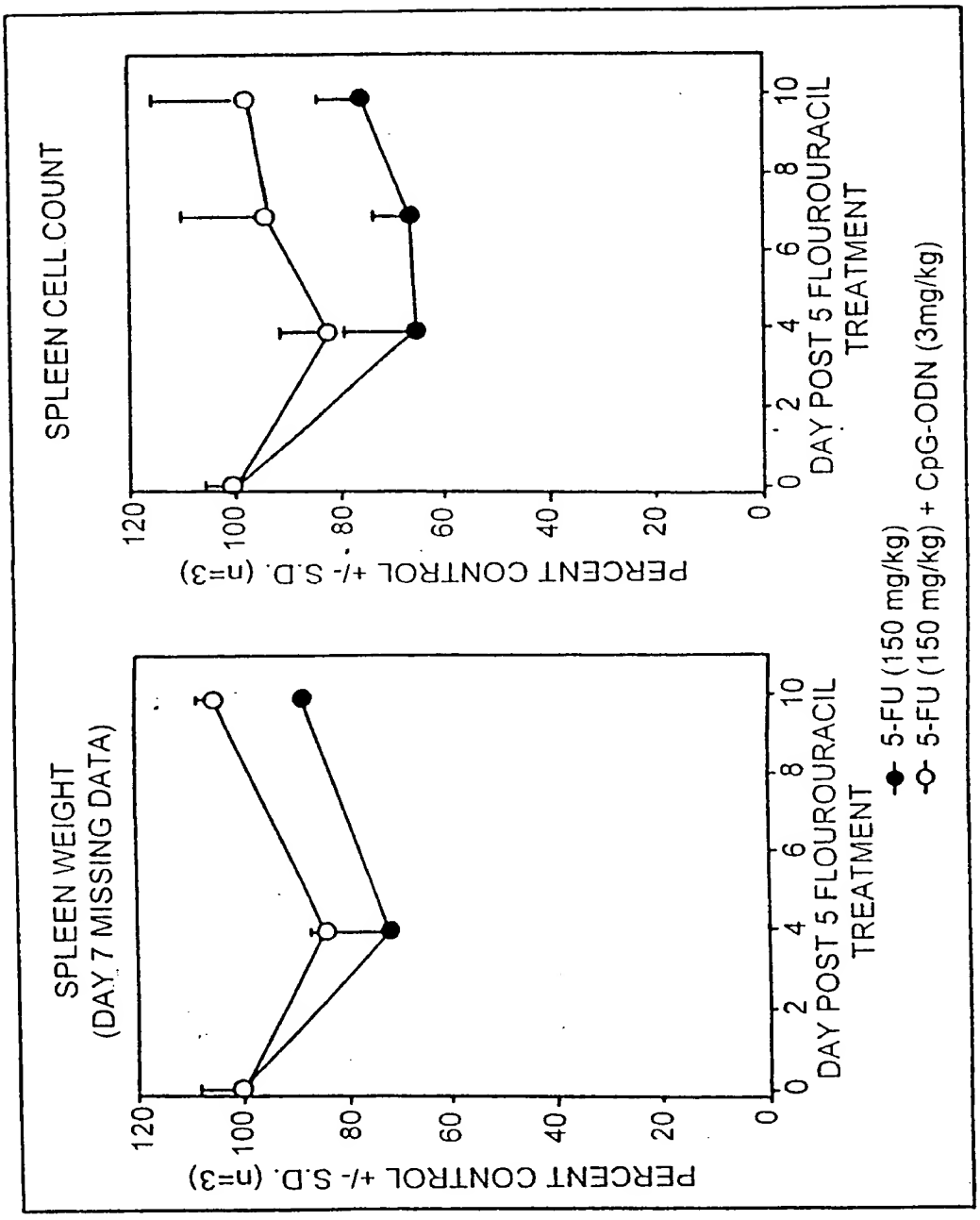


Fig. 7C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(DAY 7 MISSING DATA)
PERCENT CONTROL +/- S.D. (n=3)
DAY POST 5 FLOUROURACIL TREATMENT
SPLEEN CELL COUNT
PERCENT CONTROL +/- S.D. (n=3)
DAY POST 5 FLOUROURACIL TREATMENT
5-FU (150 mg/kg)
5-FU (150 mg/kg) + CpG-ODN (3mg/kg)

Fig. 8

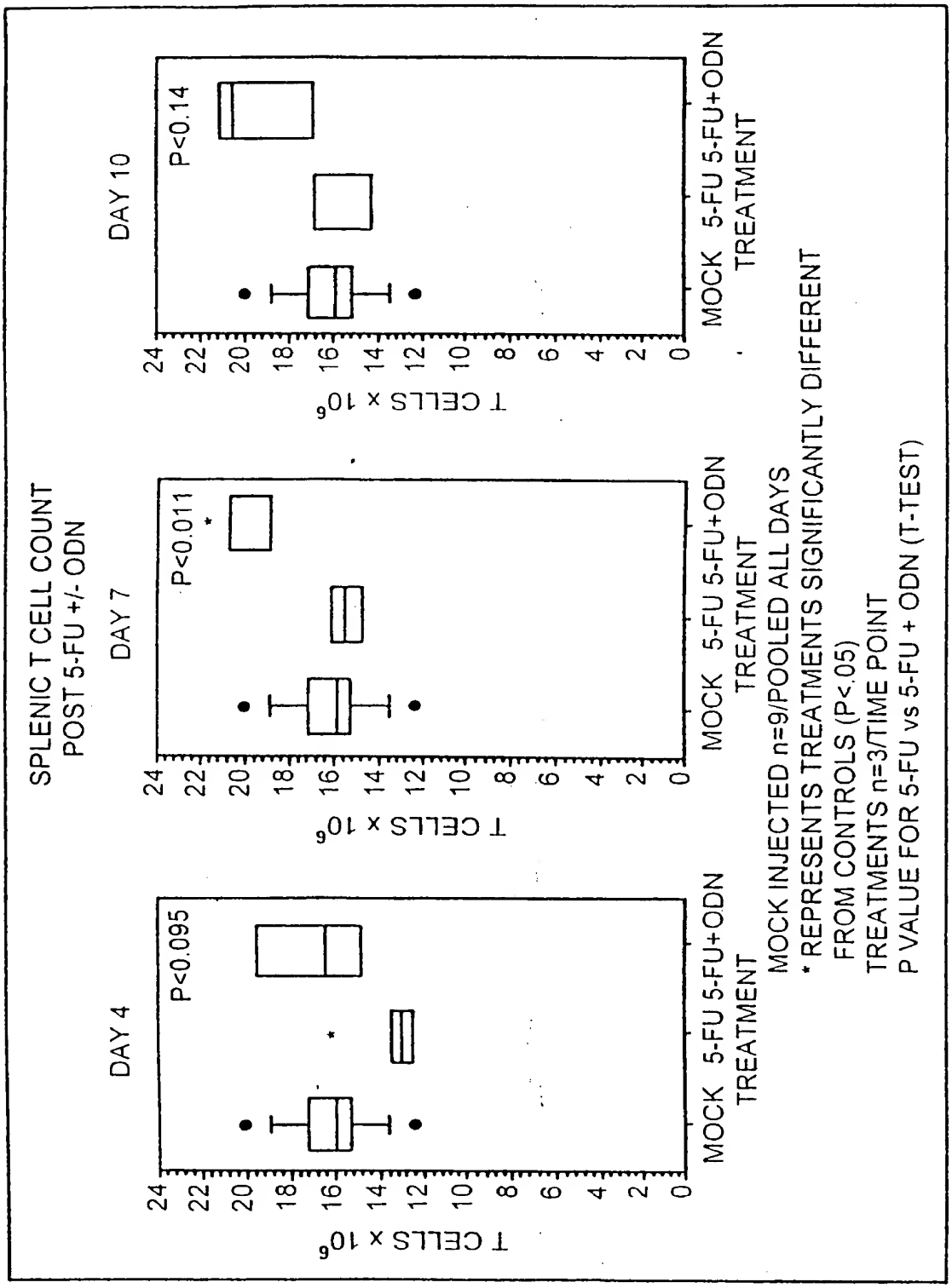
SPLENIC T CELL COUNT
POST 5-FU +/- ODN
DAY 4
P<0.095
DAY 7
P<0.011
DAY 10
P<0.14
T CELLS x 10^9
0
2
4
6
8
10
12
14
16
18
20
22
24
MOCK 5-FU 5-FU+ODN
TREATMENT
MOCK INJECTED n=9/POOLED ALL DAYS
* REPRESENTS TREATMENTS SIGNIFICANTLY DIFFERENT
FROM CONTROLS (P<.05)
TREATMENTS n=3/TIME POINT
P VALUE FOR 5-FU vs 5-FU + ODN (T-TEST)

Fig. 9

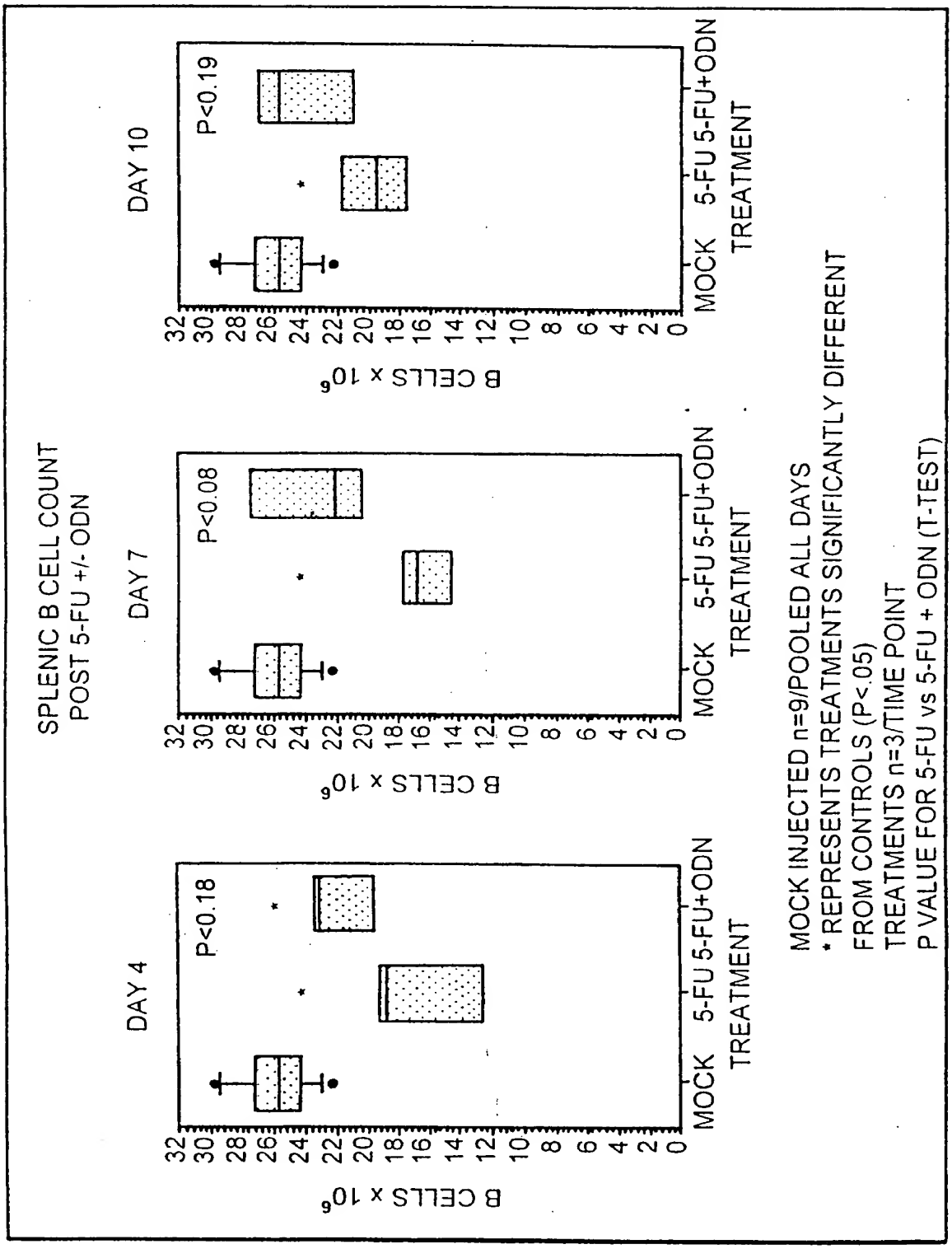
SPLENIC B CELL COUNT
POST 5-FU +/- ODN
DAY 4
P<0.18
DAY 7
P<0.08
DAY 10
P<0.19
B CELLS x 10^6
32 30 28 26 24 22 20 18 16 14 12 10 8 6 4 2 0
MOCK 5-FU 5-FU+ODN
TREATMENT
MOCK INJECTED n=9/POOLED ALL DAYS
* REPRESENTS TREATMENTS SIGNIFICANTLY DIFFERENT
FROM CONTROLS (P<.05)
TREATMENTS n=3/TIME POINT
P VALUE FOR 5-FU vs 5-FU + ODN (T-TEST)

Fig. 10

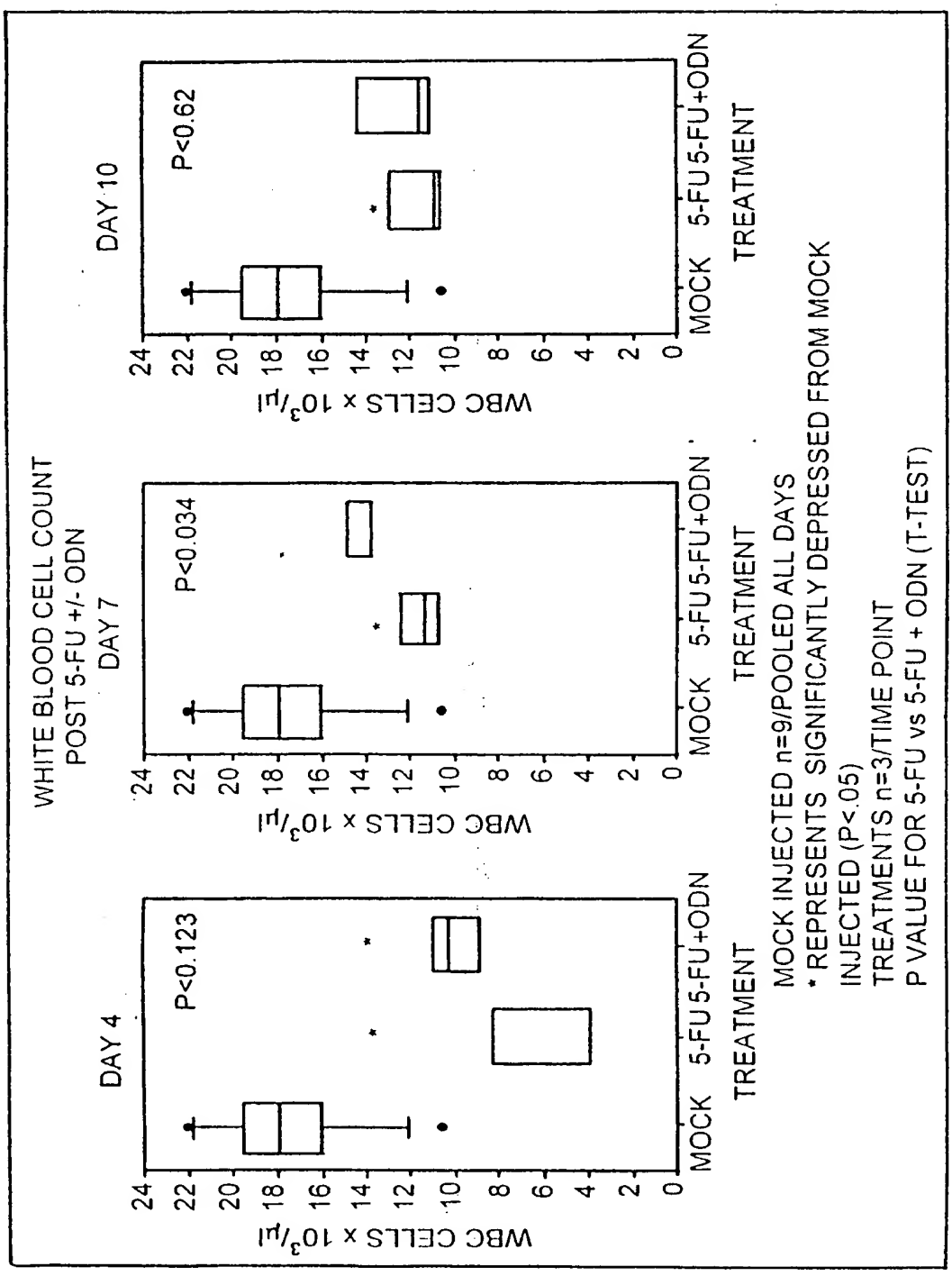
WHITE BLOOD CELL COUNT
POST 5-FU +/- ODN
DAY 4
P<0.123
DAY 7
P<0.034
DAY 10
P<0.62
WBC CELLS x 10³/µl
0
2
4
6
8
10
12
14
16
18
20
22
24
MOCK
5-FU
5-FU+ODN
TREATMENT
MOCK INJECTED n=9/POOLED ALL DAYS
* REPRESENTS SIGNIFICANTLY DEPRESSED FROM MOCK INJECTED (P<.05)
TREATMENTS n=3/TIME POINT
P VALUE FOR 5-FU vs 5-FU + ODN (T-TEST)

Fig. 11

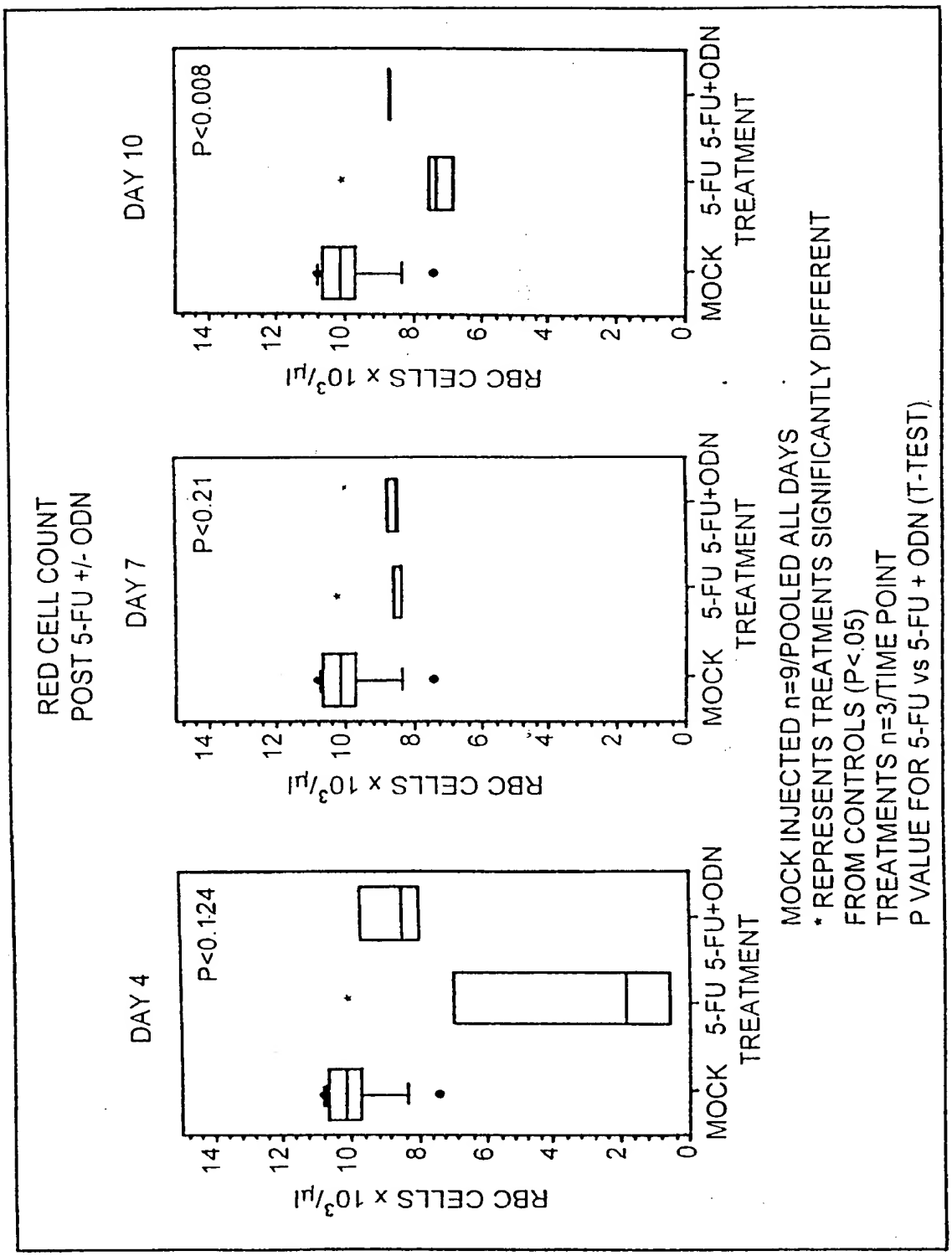
RED CELL COUNT
POST 5-FU +/- ODN
DAY 4
P<0.124
DAY 7
P<0.21
DAY 10
P<0.008
RBC CELLS x 10³/µl
0
2
4
6
8
10
12
14
MOCK
5-FU
5-FU+ODN
TREATMENT
MOCK INJECTED n=9/POOLED ALL DAYS
* REPRESENTS TREATMENTS SIGNIFICANTLY DIFFERENT
FROM CONTROLS (P<.05)
TREATMENTS n=3/TIME POINT
P VALUE FOR 5-FU vs 5-FU + ODN (T-TEST)

Fig. 12

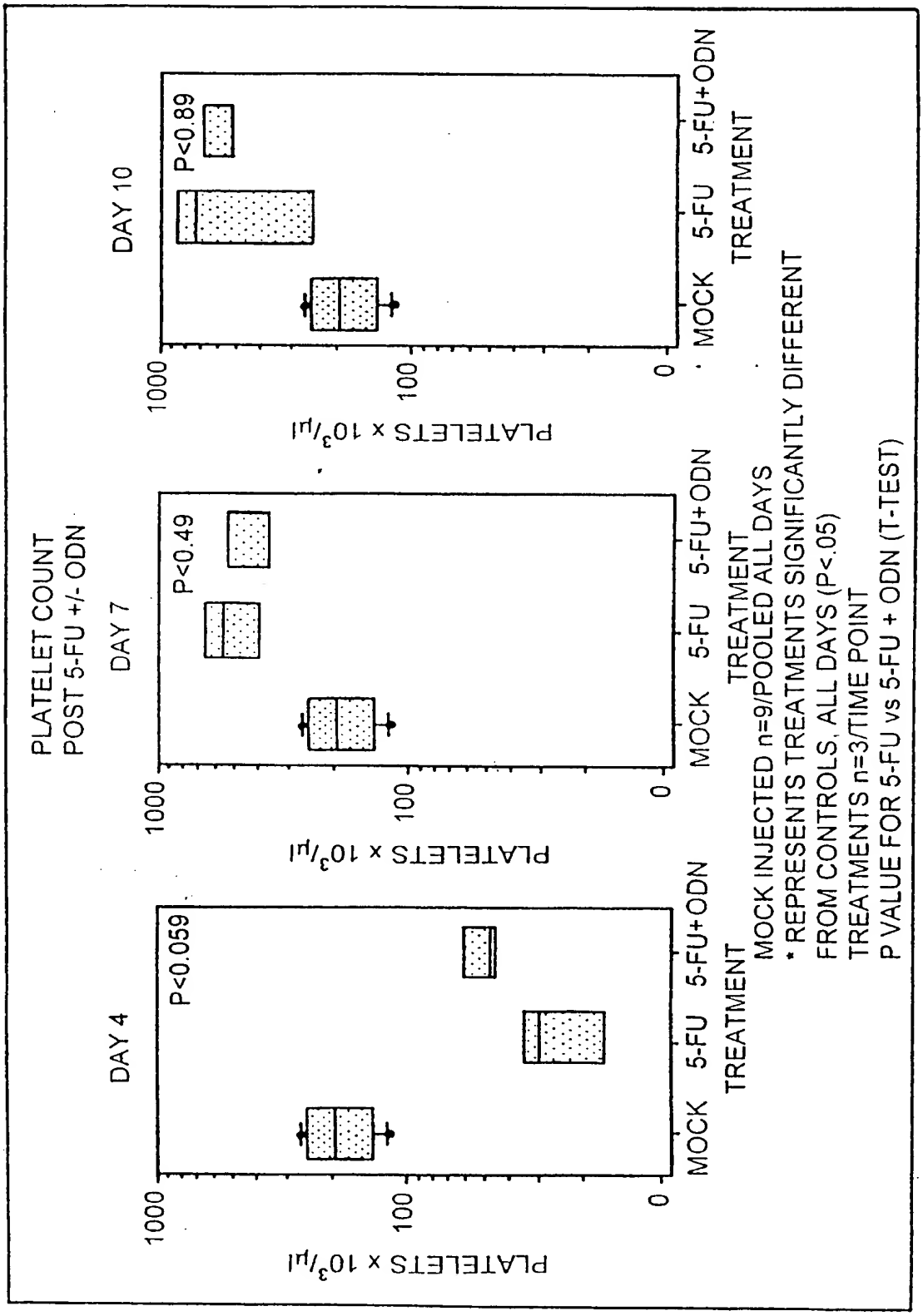
PLATELET COUNT
POST 5-FU +/- ODN
DAY 4
P<0.059
DAY 7
P<0.49
DAY 10
P<0.89
PLATELETS x 10³/µl
1000
100
0
MOCK
5-FU
5-FU+ODN
TREATMENT
MOCK INJECTED n=9/POOLED ALL DAYS
* REPRESENTS TREATMENTS SIGNIFICANTLY DIFFERENT
FROM CONTROLS, ALL DAYS (P<.05)
TREATMENTS n=3/TIME POINT
P VALUE FOR 5-FU vs 5-FU + ODN (T-TEST)

Fig. 13

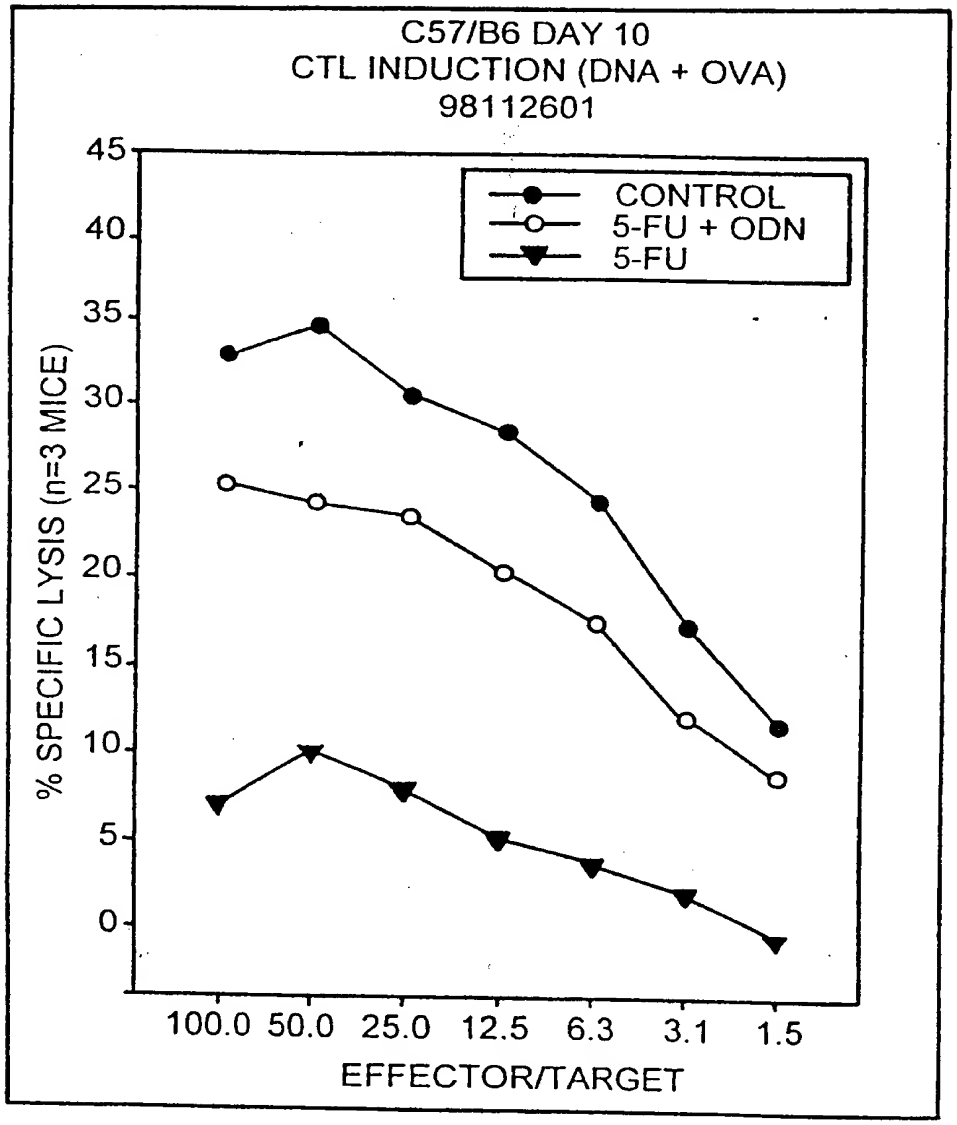
C57/B6 DAY 10
CTL INDUCTION (DNA + OVA)
98112601
CONTROL
5-FU + ODN
5-FU
% SPECIFIC LYSIS (n=3 MICE)
45
40
35
30
25
20
15
10
5
0
100.0 50.0 25.0 12.5 6.3 3.1 1.5
EFFECTOR/TARGET

Fig. 14

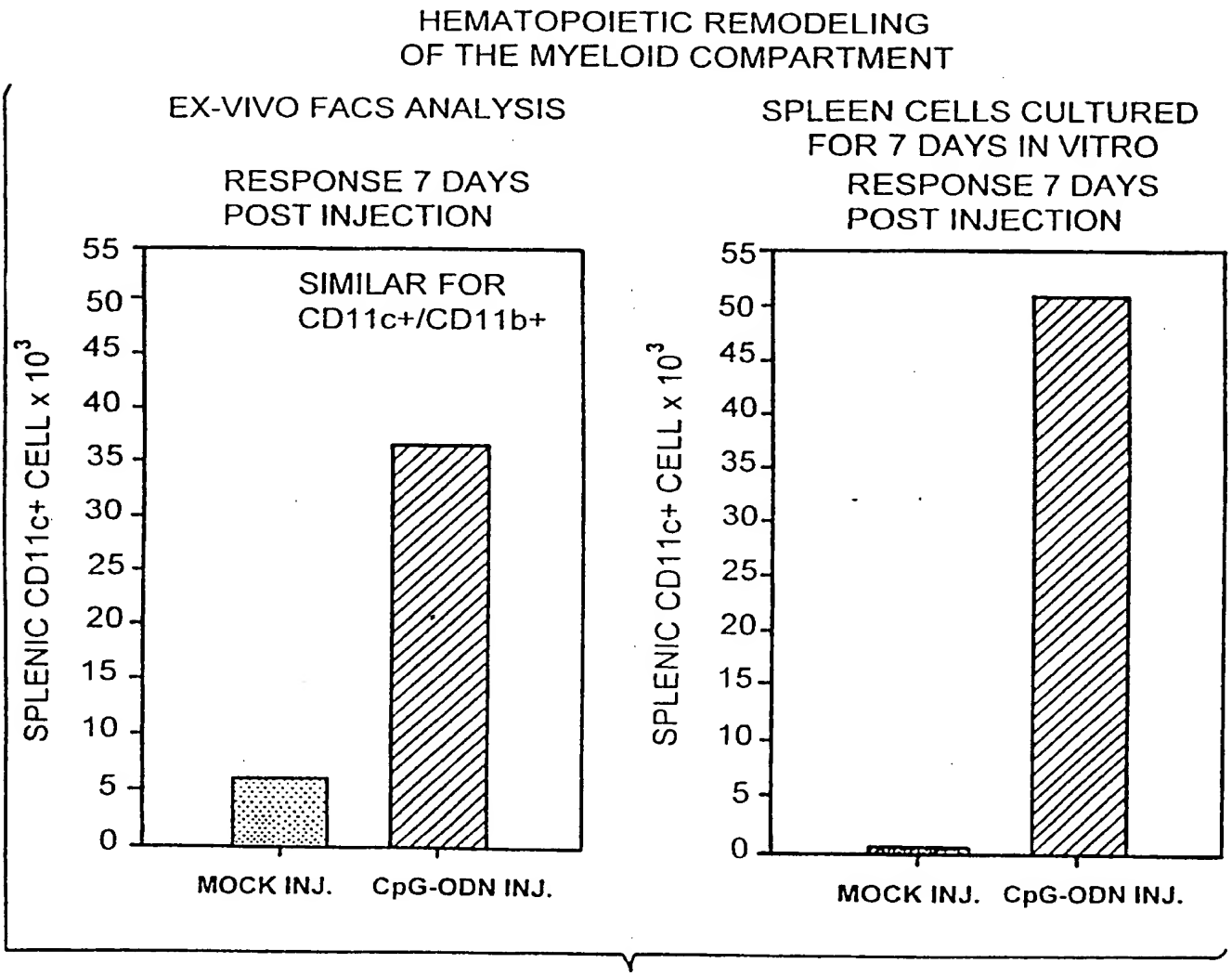
HEMATOPOIETIC REMODELING
OF THE MYELOID COMPARTMENT
EX-VIVO FACS ANALYSIS
RESPONSE 7 DAYS
POST INJECTION
SIMILAR FOR
CD11c+/CD11b+
SPLENIC CD11c+ CELL x 10³
55
50
45
40
35
30
25
20
15
10
5
0
MOCK INJ.
CpG-ODN INJ.
SPLEEN CELLS CULTURED
FOR 7 DAYS IN VITRO
RESPONSE 7 DAYS
POST INJECTION
SPLENIC CD11c+ CELL x 10³
MOCK INJ.
CpG-ODN INJ.

Fig. 15

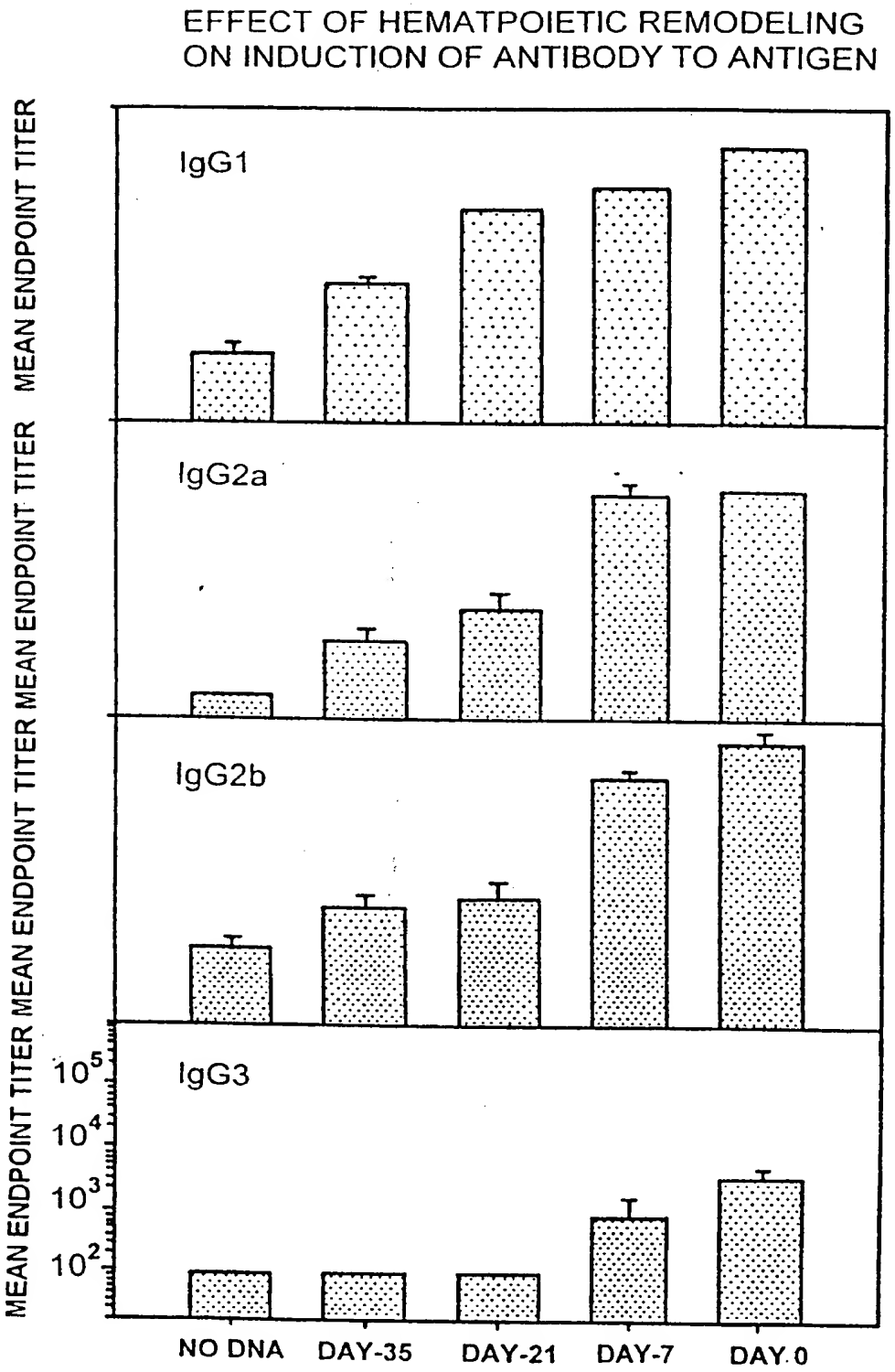
EFFECT OF HEMATPOIETIC REMODELING
ON INDUCTION OF ANTIBODY TO ANTIGEN
MEAN ENDPOINT TITER
MEAN ENDPOINT TITER MEAN ENDPOINT TITER MEAN ENDPOINT TITER
IgG1
IgG2a
IgG2b
IgG3
10^5
10^4
10^3
10^2
NO DNA
DAY-35
DAY-21
DAY-7
DAY 0

Fig. 16

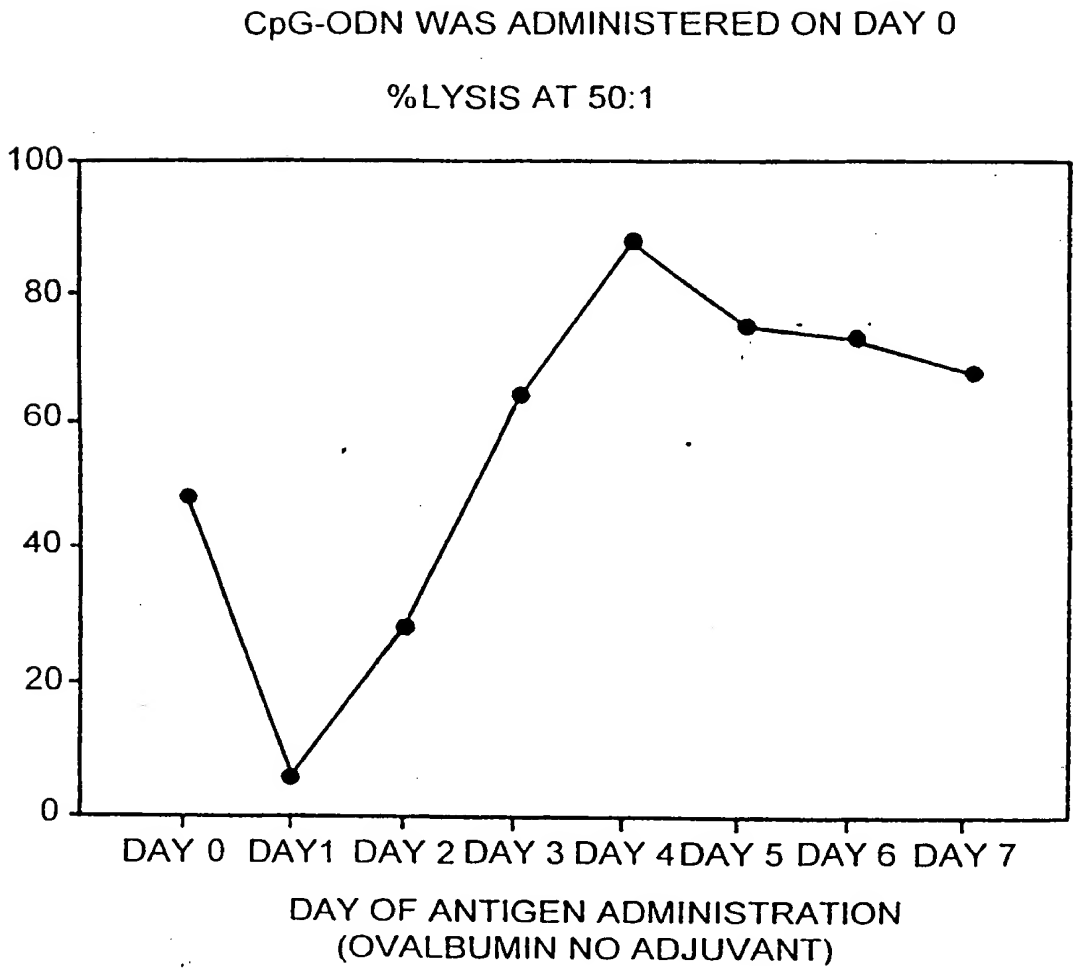
CpG-ODN WAS ADMINISTERED ON DAY 0
%LYSIS AT 50:1
100
80
60
40
20
0
DAY 0
DAY1
DAY 2
DAY 3
DAY 4
DAY 5
DAY 6
DAY 7
DAY OF ANTIGEN ADMINISTRATION
(OVALBUMIN NO ADJUVANT)

Fig. 17

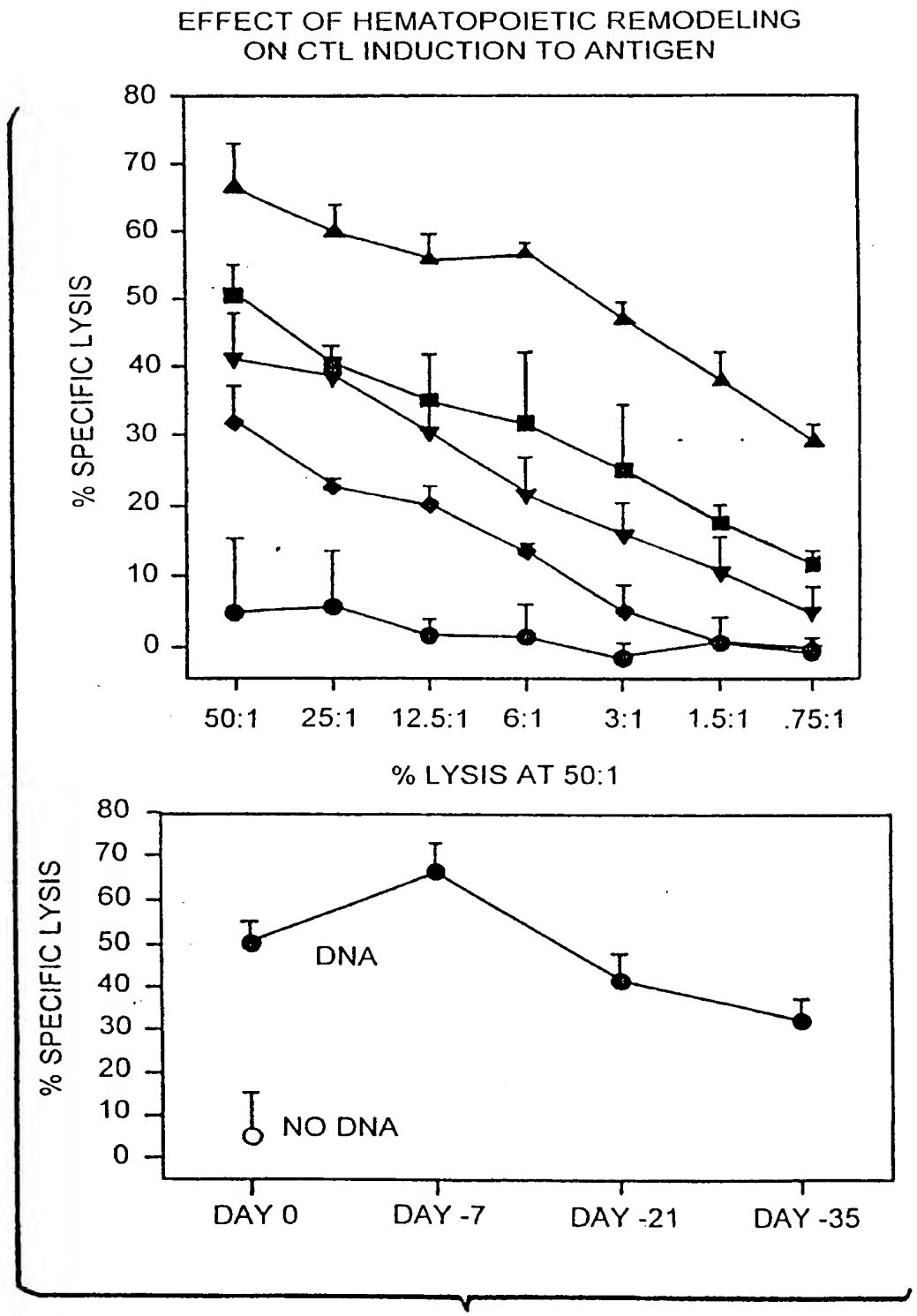
EFFECT OF HEMATOPOIETIC REMODELING
ON CTL INDUCTION TO ANTIGEN
% SPECIFIC LYSIS
80
70
60
50
40
30
20
10
0
50:1
25:1
12.5:1
6:1
3:1
1.5:1
.75:1
% LYSIS AT 50:1
% SPECIFIC LYSIS
DNA
NO DNA
DAY 0
DAY -7
DAY -21
DAY -35

Fig. 18

SEQUENCE LISTING

<110> CpG ImmunoPharmaceuticals GmbH

<120> Methods for Regulating Hematopoiesis
Using CpG- Oligonucleotides

<130> C1041/7002/WO/HCL

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EXHIBIT 2

Immunostimulatory Oligodeoxynucleotides Containing CpG Motifs Enhance the Efficacy of Monoclonal Antibody Therapy of Lymphoma

By James E. Wooldridge, Zuhair Ballas, Arthur M. Krieg, and George J. Weiner

Bacterial DNA and synthetic oligodeoxynucleotides containing the CpG motif (CpG ODN) can activate various immune cell subsets, including natural killer cells and macrophages. We evaluated whether the combination of CpG ODN and antitumor monoclonal antibody is effective at preventing tumor growth in an immunocompetent murine lymphoma model. CpG ODN–activated murine splenocytes induced lysis of tumor targets more effectively than unactivated splenocytes. These effector cells were also superior to unactivated splenocytes or cells activated with a control methylated ODN at inducing antibody-mediated lysis of 38C13 murine lymphoma cells. In vivo, CpG ODN alone had no effect on survival of mice inoculated with 38C13 cells. However, a single injection of CpG ODN enhanced the antitumor response to antitumor monoclonal antibody therapy. Ninety percent of mice treated with monoclonal antibody alone developed tumor compared with 20% of mice treated with antibody and CpG ODN. These antitumor effects were less pronounced when treatment consisted of an identical ODN containing methylated CpG dinucleotides. A single dose of CpG ODN appeared to be as effective as multiple doses of interleukin-2 at inhibiting tumor growth when combined with antitumor monoclonal antibody. We conclude that immunostimulatory CpG ODN can enhance antibody dependent cellular cytotoxicity and warrant further evaluation as potential immunotherapeutic reagents in cancer.
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RECENT STUDIES have demonstrated that bacterial DNA, but not vertebrate DNA, can have significant immunostimulatory effects on B cells,[1-3] natural killer (NK) cells,[4-6] and macrophages.[7] NK cells can be activated by the nucleic acid fraction of attenuated mycobacterial cells (Bacillus-Calmette-Guerin [BCG])[8] and by DNA synthesized in 30mer and 45mer lengths from the BCG genome. Such activated NK cells produce interferon-γ (IFN-γ) and show enhanced NK cytolytic activity.[6] NK cells activated by bacterial DNA have in vivo antitumor activity as well.[9] Recent studies demonstrate that macrophages are activated by the bacterial DNA that is internalized after phagocytosis.[7] These immunostimulatory effects are due, at least in part, to sequences containing unmethylated CpG dinucleotides, because the effects are abolished by cytosine methylation.[3] In addition, synthetic oligodeoxynucleotides containing CpG motifs (CpG ODN) also can enhance NK and macrophage activity.[4,7] Vertebrate DNA, but not bacterial DNA, exhibits CpG suppression and extensive methylation of cytosines in CpG dinucleotides. We therefore have postulated that lymphocyte activation by unmethylated CpG dinucleotides may have evolved as a mechanism by which the immune response to microbial infection could be enhanced.[3]

The immune response normally involves the integrated production of a variety of cytokines that work in concert both locally and systemically. At the cellular level, CpG ODN induce secretion of interleukin-6 (IL-6), IL-12, and IFN-γ[2,4,9] but not IL-2.[4] In unfractionated spleen cultures, NK activation by CpG ODN is unaffected by removal of B cells or T cells, but decreases markedly upon neutralization of IL-12, tumor necrosis factor-α (TNF-α), and IFN-γ.[4] Interestingly, highly purified NK cells are not activated by CpG ODN.[4,9] It therefore appears that stimulation of an additional population of cells, such as monocytes/macrophages, by CpG ODN is important in achieving NK activation.

The mechanisms responsible for CpG ODN–induced immune activation are under intensive investigation. Stacey et al[7] have found that bacterial DNA and CpG ODN can activate transcription factor nuclear factor-κB, which subsequently leads to induction of other genes involved in inflammation.[7] Preliminary data from our laboratory also suggest that DNA containing the CpG dinucleotide functions by inducing generation of reactive oxygen species.[10]

Both NK cells and monocytes/macrophages participate in antibody-dependent cellular cytotoxicity (ADCC). Activation of NK cells with cytokines can enhance ADCC in vitro and the efficacy of monoclonal antibody (MoAb) therapy in vivo.[11] Enhancement of tumor immunity may be most effective if agents are used that can orchestrate the immune response, including cytokine production and cellular activation, in a manner that reflects physiological responses. CpG ODN are such agents. The current studies were designed to evaluate whether CpG ODN can enhance ADCC in vitro and improve the therapeutic efficacy of antitumor MoAb therapy in vivo.

MATERIALS AND METHODS

Tumor model. The 38C13 murine B-cell lymphoma model has been used extensively in studies of antitumor MoAb therapy.[12,13] 38C13 cells were grown in RPMI 1640 supplemented with 100 U/mL of penicillin and streptomycin, 2-mercaptoethanol, and 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) that was heat inactivated at 56°C (complete media). Cells were used in log-phase growth. The idiotype expressed by the surface IgM of 38C13 serves as a highly specific tumor antigen. The IgG2a anti-Idiotype MoAb designated MS11G6 has been previously described[14,15] and is referred to as antitumor MoAb. It was purified from cell culture supernatant by affinity chromatography using protein A and used in both the in vitro and in vivo assays.

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Fig 1. Sequences of CpG ODN and methylated control ODN. Underlined cytosines in the control ODN were methylated.

CpG ODN 5' TCT CCC AGC GTG CGC CAT 3'

Control Methylated ODN 5' TCT CCC AGC GTG CGC CAT 3'

Oligodeoxynucleotide preparation. Phosphorothioate oligodeoxynucleotides were kindly provided by Genta (San Diego, CA) or purchased from Oligos etc (Wilsonville, OR). An immunostimulatory CpG oligodeoxynucleotide (with the sequence 5' TCT CCC AGC GTG CGC CAT 3') was selected for further study when it was found to have a significant effect on murine NK cells with little effect on murine B cells. This CpG oligodeoxynucleotide (henceforth referred to as CpG ODN) at concentrations ranging from 1.25 to 20 μg/mL also had no effect on the in vitro proliferation of 38C13 cells (data not shown). It should be noted that this sequence is also antisense to the human Bcl-2 gene and has partial homology to the antisense to mouse Bcl-2 gene. The control oligodeoxynucleotide (control methylated ODN) had an identical sequence, but contained methyloytosines in the CpG motifs (see Fig 1). Lipopolysaccharide levels were assessed by the Limulus assay and were always less than 12.5 ng/mg of ODN.

In vitro chromium release assay. A standard chromium release assay was used to detect cytotoxicity. Spleens of naive 6 to 8-week-old C3H/HeN mice were homogenized, and erythrocytes were removed by hypo-osmolar lysis with a solution containing 0.15 mol/L NH_4Cl, 1.0 mmol/L $KHCO_3$, 0.1 mmol/L Na_2EDTA at a pH of 7.4. B cells were removed by anti-Ig selection using a biotinylated goat antimouse Ig (Sigma, St Louis, MO) and avidin-coated micromagnetic beads eluted over a magnetic column (Miltenyi Biotech, Auburn, CA). The eluted cells were washed with media and cultured in complete media alone or media supplemented with CpG ODN, control methylated ODN, or 500 U/ml. of recombinant human IL-2 (Chiron, Emeryville, CA) for 24 to 72 hours before use as effector cells. Target cells (38C13) were labeled with 200 μCi of Cr^{51} (Amersham Life Sciences, Arlington Heights, IL) for 1 hour and washed three times. Cells were divided and incubated at 4°C for 30 minutes in complete media or media supplemented with 5 μg/mL antitumor MoAb before distribution into 96-well V-bottom culture plates (Costar, Cambridge, MA) at the desired cellular concentrations. Effector cells were added at the desired effector to target ratios. Plates were incubated for 4 hours at 37°C in a humidified environment containing 5% CO_2 and supernatants evaluated by γ counting. Nonspecific release of Cr^{51} (percent release from target cells incubated for 4 hours without effector cells or MoAb) was less than 20% for all experiments. Samples were run in triplicate, and the percentage of lysis was determined.

In vivo tumor therapy. 38C13 cells grow rapidly and consistently in syngeneic, immunocompetent C3H/HeN mice. Female C3H/HeN mice, obtained from Harlan-Sprague-Dawley (Indianapolis, IN) and housed in the Animal Care Unit at the University of Iowa, were used at 6 to 8 weeks of age. Mice were inoculated with 2,500 38C13 tumor cells intraperitoneally (IP), and therapy with CpG or control methylated ODN, IL-2, and antitumor MoAb was administered IP beginning 2 days later as indicated. CpG ODN and control methylated ODN were used at a dose of 300 μg per mouse because pilot studies demonstrated systemic effects at that dose. Survival was determined, and significance with respect to time to death was assessed using Cox regression analysis.[16] Mice were observed daily for signs of toxicity including level of activity, ruffled fur, diarrhea, and general appearance.

RESULTS

CpG ODN enhances NK cytolytic activity and ADCC in vitro. We first assessed the NK cytolytic activity of CpG ODN–stimulated effector cells in vitro using NK-sensitive YAC-1 cells as targets as previously reported.[4] Effector cells stimulated with IL-2 served as a control. Unstimulated effector cells and effector cells stimulated with 2.5 μg/mL of control methylated ODN induced minimal lysis, whereas significant lysis was mediated by effector cells stimulated with 2.5 μg/mL of CpG ODN or IL-2 at a concentration of 500 U/mL (data not shown). We then explored whether CpG ODN enhances ADCC in vitro. As illustrated in Fig 2, lysis of 38C13 cells was minimal when unstimulated effector cells or effector cells stimulated with control methylated ODN were used. IL-2–activated effector cells were capable of mediating lysis. This was enhanced further when antitumor MoAb was added. Similar, although less profound effects were noted when CpG ODN-stimulated effector cells were used. Thus, CpG ODN enhanced ADCC mediated by antitumor MoAb, but not to the same degree as IL-2.

CpG ODN enhances antitumor therapy with antitumor MoAb. Previous studies have demonstrated that antitumor MoAb in the 38C13 model has a moderate antitumor effect when administered in large doses (100 μg) 4 hours after inoculation of the mice with tumor. The antitumor effect of MoAb is limited in this rapidly growing tumor model when therapy is delayed for greater than 24 to 48 hours after tumor inoculation.[14,15,17] We therefore assessed whether treatment with CpG ODN allows for more effective MoAb therapy when treatment with MoAb is delayed beyond the time when

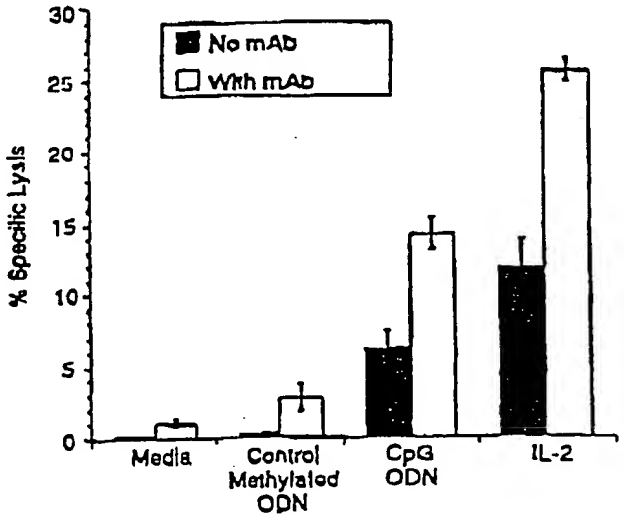

Fig 2. CpG ODN or IL-2 prestimulation of mononuclear splenocytes in combination with MoAb enhances lysis of 38C13 cells in vitro. Splenocytes were activated as described in the Materials and Methods. Target 38C13 cells were labeled with chromium-51 for 1 hour followed by incubation with MoAb (5 μg/mL) for 30 minutes at 4°C. Effector cells and target cells were plated at a 25:1 effector-target ratio and chromium release was determined.

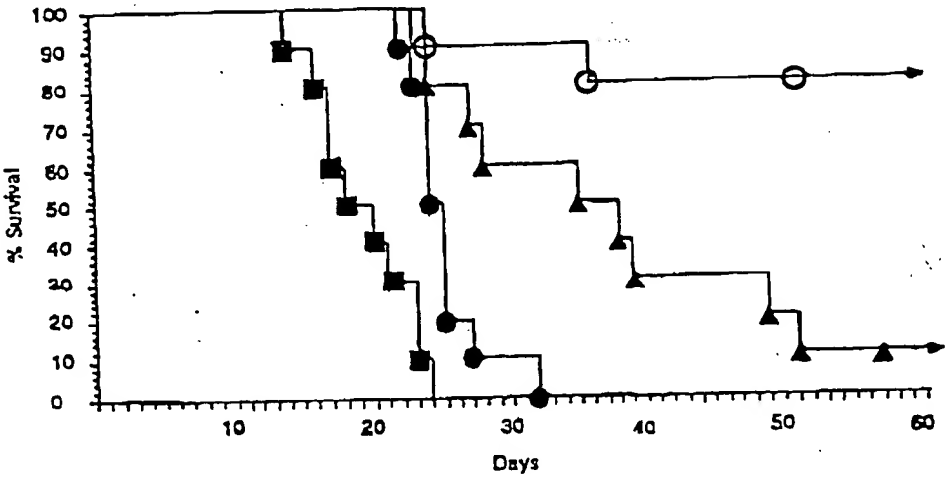

Fig 3. CpG ODN and MoAb are more effective than MoAb alone in vivo at preventing death from tumor. Immunocompetent C3H mice were inoculated with 2,500 tumor cells IP on day 0. Therapy consisted of (■) no ODN or MoAb, (▲) MoAb alone, (●) CpG ODN alone, or (○) CpG ODN and MoAb. CpG ODN (300 μg) was administered IP on day 2, and MoAb (50 μg) was administered on day 3. Each group contained 10 mice. Curves depict percent survival over 60 days. No mice living at day 60 developed tumor, and all survived more than 8 months. No toxicity was observed in any group.

MoAb is usually effective (Fig 3). Mice were inoculated with 2,500 38C13 cells and divided into groups of 10. Mice received no therapy (group 1), treatment with a single IP injection of 300 μg CpG ODN on day 2 (group 2), therapy with a single injection of 50 μg antitumor MoAb IP on day 3 (group 3), or both CpG ODN and antitumor MoAb (group 4). No toxicity was noted in any group. All mice in the control group (group 1) died within 24 days. Mice that received CpG ODN alone (group 2) had slightly improved survival, but all developed lymphoma and died within 34 days. Mice treated with antitumor MoAb alone had prolonged survival when compared with untreated mice, but only 1 of 10 mice achieved long-term survival. In contrast, 8 of 10 mice treated with CpG ODN followed by antitumor MoAb survived without evidence of tumor. The survival of this group was highly significant when compared with the other groups (v CpG ODN alone, $P < .001$; v MoAb alone, $P = .0109$).

Comparison of antitumor therapy with CpG ODN and antitumor MoAb to therapy with IL-2 and antitumor MoAb. IL-2 has been shown to enhance the efficacy of anti-idiotype MoAb therapy in the 38C13 model. IL-2 is most effective when administered repeatedly.[11] We therefore compared in vivo therapy of antitumor MoAb with CpG ODN to therapy with MoAb and IL-2 (Fig 4). Mice in the IL-2 groups received 50,000 U IL-2 twice daily for 3 days. Mice received no therapy (group 1), treatment with a single IP injection of 300 μg CpG ODN on day 2 (group 2), therapy with a single injection of 50 μg antitumor MoAb IP on day 3 (group 3), both CpG ODN and antitumor MoAb (group 4), IL-2 alone (group 5), or IL-2 and MoAb (group 6). CpG DNA plus antitumor MoAb therapy resulted in long-term survival of 70%, whereas IL-2 plus antitumor MoAb resulted in long-term survival of only 40%. This difference did not reach statistical significance ($P = .20$).

Comparison of antitumor therapy with CpG ODN and antitumor MoAb to therapy with control methylated ODN and antitumor MoAb. In vitro studies suggested the control methylated ODN had little effect on enhancement of ADCC. However, it was possible other mechanisms, such as antisense activity, contributed to the antitumor effect seen in vivo with CpG ODN and MoAb. We therefore evaluated therapy in vivo with MoAb and control methylated ODN in parallel with the IL-2 study outlined above. Control methylated ODN added little to the therapeutic efficacy of MoAb alone (control methylated ODN and MoAb v MoAb alone, $P = .12$) (Fig 5) in contrast to the studies outlined in Fig 3 where therapy with MoAb and CpG ODN was more effective than therapy with MoAb alone.

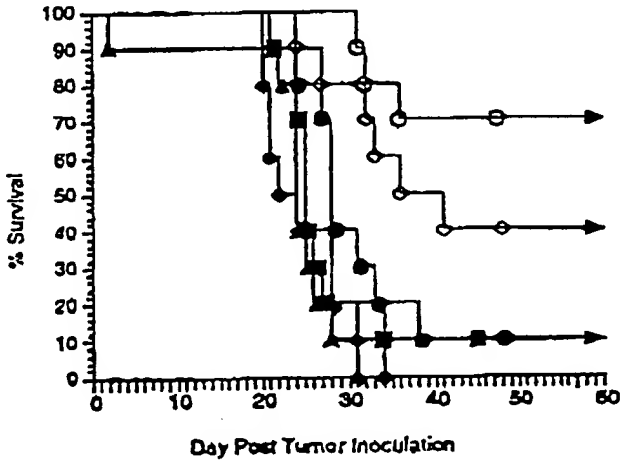

Fig 4. Therapy with CpG ODN and MoAb enhances survival as effectively as therapy with IL-2 and MoAb. Immunocompetent C3H mice were inoculated with 2,500 tumor cells IP on day 0. Therapy consisted of MoAb alone, CpG ODN alone, IL-2 alone, IL-2 and MoAb, or CpG ODN and MoAb. IL-2 (50,000 U) was administered IP twice daily on days 2, 3, and 4. CpG ODN (300 μg) was administered IP on day 2, and MoAb (50 μg) was administered on day 3 as in Fig 3. Each group contained 10 mice. Curves depict the percentage of survival over 60 days. No mice living at day 60 developed tumor, and all survived more than 8 months. No toxicity was observed in any group. (■) No therapy; (●) MoAb on day 3; (▲) CpG ODN on day 2; (◆) IL-2 on days 2, 3, and 4; (○) IL-2 on days 2, 3, and 4 and MoAb on day 3; (◇) CpG ODN on day 2 and MoAb on day 3.

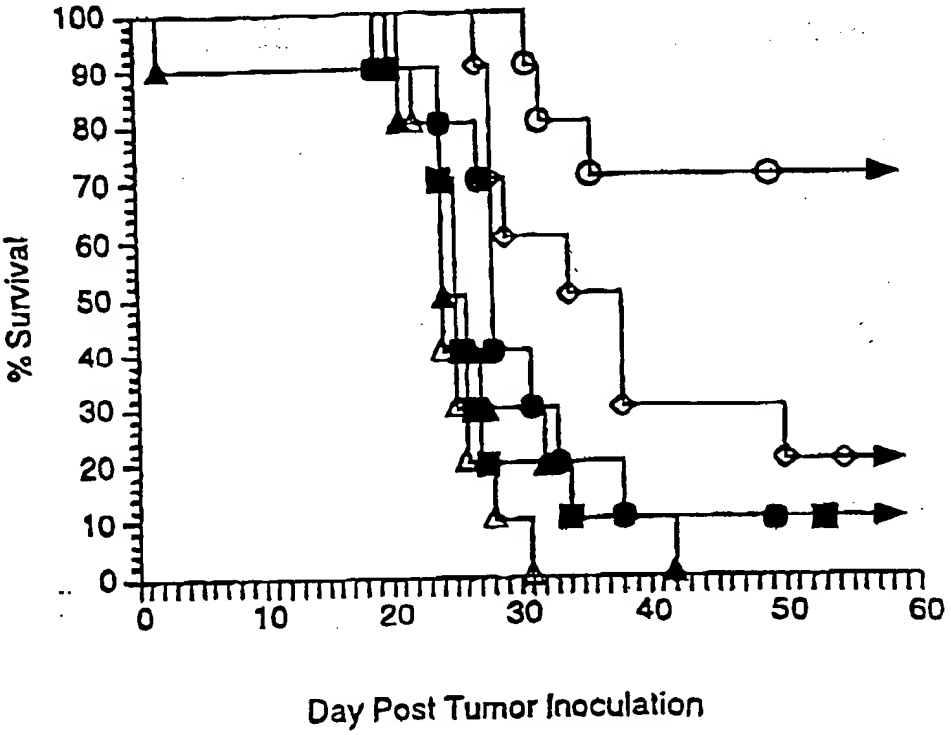

Fig 5. Therapy with CpG ODN and MoAb enhances survival more effectively than therapy with control methylated ODN and MoAb. Immunocompetent C3H mice were inoculated with 2,500 tumor cells IP on day 0. Therapy consisted of MoAb alone, control methylated ODN alone, CpG ODN alone, control methylated ODN and MoAb, or CpG ODN and MoAb. Mice were followed for tumor development, toxicity, and survival. Each group contained 10 mice. Curves depict percent survival over 60 days. No mice living at day 60 developed tumor, and all survived more than 5 months. No toxicity was observed in any group. (■) No therapy; (●) MoAb on day 3; (▲) control methylated ODN on day 2; (△) CpG ODN on day 2; (○) control methylated ODN on day 2 and MoAb on day 3; (○) CpG ODN on day 2 and MoAb on day 3.

DISCUSSION

B-cell lymphomas are among the most sensitive tumors to MoAb-based immunotherapy. Agents known to enhance NK activity, such as IL-2, have been shown to enhance the antitumor effects of antilymphoma MoAb. We used a well-established lymphoma model to evaluate whether the antitumor effects of MoAb can be enhanced by CpG ODN that induce NK activation. There was clear synergy between CpG ODN and antitumor MoAb in this model and the most likely explanation for this finding is enhanced ADCC. It is unlikely that the CpG ODN has a direct effect on tumor cells, given tumor proliferation was not inhibited in vitro by CpG ODN and only minimal therapeutic benefit was seen in the group treated with CpG ODN alone. An alternative explanation is that the CpG ODN-induced production of cytokines rendered the tumor cells more sensitive to ADCC.

These effects were largely eliminated when an ODN containing identical sequences but having methylated cytosines in the CpG motif was used. Because the 5-methyl cytosine base substitutions actually increase the affinity for hybridization to complementary mRNA, this finding demonstrates that the antitumor effect of this ODN cannot be attributed solely to an antisense mechanism.

Anecdotal reports of tumor regression after systemic bacterial infection have been observed for centuries. Experimental antitumor therapy with heat-inactivated bacteria was reported by Dr William Coley[18] in the 1890s. Dr Coley's original attempt to use bacteria as an antitumor agent involved the use of live cultures of streptococci.[18] This resulted in tumor regression, but proved to be toxic with the first patient almost dying of erysipelas. Subsequent studies by Coley involved a mixture of heat-killed *Streptococci* and *Serratia* (then known as *Bacillus prodigiosus*).[19] It was this preparation that is now known as Coley's toxin. Much of the antitumor activity of Coley's toxin is currently attributed to endotoxin. A number of cytokines induced by endotoxin (such as TNF-α and IFN-γ) have been produced in recombinant form and have been shown to have antitumor activity.[20] However, it is curious to note that Coley's original success was with an organism that does not produce endotoxin. It is possible bacterial DNA with its unmethylated CpG motifs played a role in the antitumor effects seen in Coley's original preparation. Whether the responses seen by Dr Coley were related to the immunostimulatory effects of streptococcal DNA, the data presented above indicate that motifs found in bacterial DNA can have antitumor effects, particularly when used with other agents such as MoAb.

A number of important questions remain to be answered. The mechanisms by which bacterial DNA and CpG ODN interact with the cell surface of immune effector cells are internalized and induce activation signals that lead to NK cell and macrophage stimulation need to be clarified. It will also be important to understand more thoroughly how different subsets of immune cells respond to CpG ODN and what aspects of that response are related directly to CpG ODN stimulation or indirectly to cytokines such as IL-6, IL-12, or IFN. Although we observed no toxicity in vivo, the toxicity profile of CpG ODN will require definition. We detected no direct effect of the CpG ODN on 38C13 lymphoma cells; however, it is possible the CpG ODN induced changes in the tumor cells that rendered them more sensitive to MoAb

therapy. These studies therefore need to be confirmed in another tumor model and using other CpG ODN.

As we learn more about the immune system, it is becoming obvious that an effective antitumor immunotherapy will need to integrate several aspects of the immune system, including establishment of appropriate cytokine profiles, modification of tumor immunogenicity, enhancement of tumor antigen recognition, and appropriate effector cell expansion and activation. The studies outlined above demonstrate immunostimulatory DNA in general, and CpG ODN in particular, may have a role to play in the development of such a therapy.

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Pharmacokinetics of a ^{14}C-Labeled Phosphorothioate Oligonucleotide, ISIS 2105, after Intradermal Administration to Rats

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Isis Pharmaceuticals, Inc., Carlsbad, California (L.T., S.R.O., S.T.C.); Arthur D. Little, Inc., Cambridge, Massachusetts (P.M.M., J.P.S.); and Triplex Pharmaceuticals Corporation, The Woodlands, Texas (P.A.C.)

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ABSTRACT

After intradermal administration of 3.7 mg/kg of ^{14}C-labeled 5′-TTGCTTCCATCTTCCTCGTC-3′ (^{14}C-labeled ISIS 2105) to rats, a phosphorothioate oligodeoxynucleotide, absorption was rapid. Approximately 65% of the administered dose was absorbed within 1 hr after the dose and peak blood levels were achieved within 30 min. After the initial rapid phase of absorption, a slower absorption phase ensued that resulted in more than 95% of the dose being cleared from the injection site. Slow metabolism of ^{14}C-labeled ISIS 2105 occurred at the injection site. The rate and characteristics of metabolism in the skin were similar to those observed in other tissues. Once absorbed, the pharmacokinetics, distribution and metabolism of ^{14}C-labeled ISIS 2105 after intradermal administration were comparable to those after an i.v. dose. The distribution and terminal half-lives were 0.5 and 53 hr, respectively. Levels of ^{14}C-labeled ISIS 2105 in the blood were found in the plasma and the drug distributed broadly to all peripheral tissues; the liver, renal cortex and bone marrow accumulated the highest levels of drug. The ^{14}C-labeled ISIS 2105 was eliminated principally by metabolism. Approximately 50% of the dose was found in expired air and 15% and 5% were found in urine and feces, respectively. No intact oligonucleotide was found in urine or feces at any time.

Substantial interest in the development of oligonucleotide-based therapeutic agents has been generated (Zamencik and Stephenson, 1978; Stein *et al.*, 1988; Mirabelli *et al.*, 1991; Crooke, 1992). Several first-generation oligonucleotide analogs, in which one or more of the substituents on the internucleotide phosphate are modified, *e.g.*, phosphorothioates, methylphosphonates or phosphorodithioates, have been synthesized and tested (Matsukura *et al.*, 1987; Crooke, 1992; Crooke and Lebleu, 1993; Cohen, 1989).

Phosphorothioate oligodeoxynucleotides were shown to be stable (half-lives > 24 hr) in serum, cell homogenates, cells, cerebrospinal fluid and organs (Crooke, 1991; Campbell *et al.*, 1990; Agrawal *et al.*, 1988; Crooke, 1993; Stein *et al.*, 1988; Loke *et al.*, 1989; Hoke *et al.*, 1991). They are taken up by many types of cells in tissue culture (Crooke, 1991, 1993) and cellular uptake and *in vivo* activities can sometimes be enhanced by cationic lipids (Bennett *et al.*, 1992; Perlaky *et al.*, 1993).

The pharmacokinetic properties of phosphorothioate oligodeoxynucleotides in animals have been evaluated in several studies. The pharmacokinetic properties of a 20-mer phosphorothioate oligodeoxynucleotide were determined after single i.v. or i.p. doses of 30 mg/kg. The oligonucleotide was labeled at each internucleotide linkage with ^{35}S. The compound was shown to be bioavailable after i.p. administration, to have broad peripheral tissue distribution and to be cleared primarily by renal excretion. Gel electrophoretic analysis demonstrated significant, albeit slow, metabolism in the liver, kidney and intestines (Agrawal *et al.*, 1991). A 27-mer phosphorothioate deoxyoligonucleotide labeled with ^{35}S was reported to display biexponential elimination in the plasma with an elimination half-life in excess of 40 hr after a single i.v. dose of 4.8 mg/kg in the rat (Iversen, 1991).

ISIS 2105, is a phosphorothioate deoxyoligonucleotide that is active against human papillomaviruses (Cowsert *et al.*, 1993). It is currently undergoing pivotal phase II clinical trials for the treatment of genital warts. In a previous publication (Cossum *et al.*, 1993), we reported the pharmacokinetic profile of [^{14}C]2105 in rats after i.v. bolus injection. After i.v. administration, [^{14}C]2105 was extensively bound to serum albumin and α_2-macroglobulin and displayed multiexponential plasma clearance with a terminal phase of 51 hr, broad tissue distribution and elimination primarily by slow metabolism.

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ABBREVIATIONS: HPLC, high-performance liquid chromatography; ISIS 2105, 5′-TTGCTTCCATCTTCCTCGTC-3′; [^{14}C]2105, ^{14}C-labeled ISIS 2105.

Inasmuch as ISIS 2105 is currently being administered intralesionally to patients with genital warts, in the present study, we evaluated the pharmacokinetic profile of [^{14}C]2105 in rats after intradermal administration. This study provides evidence of rapid and extensive absorption of [^{14}C]2105 after intradermal administration, supports a relatively infrequent intralesional dosing schedule in patients with genital warts and provides a basis for direct comparison of pharmacokinetic profiles in rats with those in ongoing studies in humans.

Materials and Methods

Synthesis and purification of ISIS 2105. [^{14}C]2105 was chemically synthesized using the deoxynucleoside phosphoramidite approach (Beaucage and Caruthers, 1981; Matteucci and Caruthers, 1981). The phosphorothioate linkage was generated by oxidizing with 3*H*-1,2-benzodithiole-3-one-1,1-dioxide (Iyer *et al.*, 1990), instead of aqueous iodine. All reagents and materials for the solid phase synthesis of DNA were purchased from commercial sources, with the exception of the ^{14}C-labeled thymidine phosphoramidite. The ^{14}C-labeled phosphoramidite synthon was generated from ^{14}C-labeled thymidine (^{14}C at the carbon-2 position of the thymine ring; specific activity, ~56.3 mCi/mmol; Sigma, St. Louis, MO) as described elsewhere (D. Dellinger and H. Sasmor, unpublished data).

The crude synthetic oligonucleotide was purified by trityl-on reverse-phase HPLC by using a methanol gradient in a 0.25 M sodium acetate mobile-phase buffer. The HPLC product was acid deprotected and recovered by ethanol precipitation as the sodium salt. The final product was analyzed by 20% denaturing polyacrylamide gel electrophoresis and the full-length integrity (88% full-length material) and radiochemical purity (88% of the counts per minute in the full-length product) were determined by laser scanning densitometry and quantitative phosphorimaging (Molecular Dynamics, Foster City, CA), respectively. The specific activity of the final product was approximately 2.0×10^8 cpm/μmol and had approximately 97.6% phosphorothiodiester content (*vs.* 2.4% phosphodiester) as determined by high-field nuclear magnetic resonance (500 MHz, University of Missouri, Columbia, MO).

Formulation of [^{14}C]2105. [^{14}C]2105 with a specific activity of approximately 12.0 μCi/mg was diluted in phosphate-buffered saline to 1.22 mM. The solution was then sterile filtered through a 0.22-μm cellulose acetate filter (S & S Uniflow, Keene, NH), which resulted in approximately 2.3×10^8 dpm/ml.

Compound administration. Twenty-four hours before dosing, the backs of the animals were shaved and an area of approximately 10 cm^2 was demarcated. [^{14}C]2105 was administered in solution in phosphate buffer, pH 7, to rats intradermally in the demarcated area. The dosing volume was 100 μl. The concentration of [^{14}C]2105 was 1.2 mM; therefore, the dose level was approximately 3.7 mg/kg. Any material that perfused through the skin during dosing was collected by gently swabbing the injection site with absorbent material. The radioactivity, collected on the absorbent material, was extracted into a volume of water and the total amount of radioactivity was determined. The actual doses administered were calculated by subtracting the amount of radioactivity that perfused through the skin from the assayed concentration of radioactivity in the formulation.

[^{14}C]2105 was administered to 25 female rats. Five rats were used for the collection of urine, feces, expired air and tissues at the time of sacrifice. However, during dosing, it was noted that one animal received a partial dose of air intradermally. After evaluation of the recovery data, it was confirmed that this animal received an incomplete dose of [^{14}C]2105 and, therefore, data from this animal were eliminated from the analysis. The other 20 single-dose animals were used for the collection of blood at intervals after dosing and tissues at the time of sacrifice.

Animals, sample collection, quantitation of radioactivity and extraction of tissue radioactivity. These methods were the same as those described in our previous publication (Cossum *et al.*, 1993).

HPLC. Strong anion-exchange HPLC was used to determine the metabolic profile of plasma, urine, liver and kidney tissues. Ion-exchange analyses were carried out using a Beckman System Gold liquid chromatography system with model 126 pumps, model 507 autoinjector and model 166 detector (Fullerton, CA). We analyzed 40 μl of each sample of plasma and urine or of liver or kidney homogenate at 260 nm on a 4.6 × 100-mm Gen Pak Fax column (Waters, Milford, MA) by using the following buffers and gradient. Buffer A consisted of 0.086 M Tris HCl, pH 8.0, and 20% methanol. Buffer B included 0.086 M Tris HCl, pH 8.0, and 1.5 M NaBr. The gradient solution was 0% B isocratic for 5 min and then linear to 60% B over 45 min at a flow of 0.5 ml/min. Fractions (0.5 ml) were collected and added to 5 ml of Readysafe scintillation cocktail (Beckman, Fullerton, CA). Then they were counted in a Beckman model LS6000IC scintillation counter.

Plasma samples were also analyzed by size-exclusion HPLC by using a Hewlett-Packard (Pasadena, CA) model 1090 M liquid chromatography system and a 7.8 × 300-mm TSK-gel G2000 SWXL column (Tosohaas, Montgomeryville, PA). The analyses were carried out in 0.05 M Na_2HPO_4, 0.1 M Na_2SO_4 and 0.05 M NaH_2PO_4, pH 7.0, at a flow of 0.45 ml/min. On-line radiochemical detection was accomplished with a Radiomatic FLO-ONE/beta model A-525A detector (Packard Instruments, Meriden, CT). The scintillation cocktail, Ultimate-Flo-V, was purchased from Packard Instruments and was used at a flow rate of 0.9 ml/min.

Calculations. Concentrations of radioactivity in samples processed by combustion were corrected for the recovery efficiency of the combustion system, which was determined daily before the combustion of experimental samples.

The observed radioactivity values were converted to compound radioequivalent concentrations. Radioequivalents are defined as the amount of parent compound, at the specific activity as administered, that would result in the observed disintegrations per minute.

Compound equivalents in a biological sample were determined by dividing the disintegrations per minute in the sample by the specific activity of the compound in disintegrations per minute per microgram. Compound equivalents were expressed in micrograms per gram of tissue and, when possible, as a percentage of the administered dose/organ or tissue. For the purposes of calculating a mean ± S.D., tissue samples in which radioactivity was less than twice the background for the system, the equivalents were less than 0.005 μg-equivalents/g or the radioactivity was less than 0.005% of the dose were considered to have a value of zero.

The radioactivity in the urine, feces, volatile traps and cage rinse was expressed as a percentage of the administered dose for each time interval and as a cumulative percentage. For the purposes of calculating a mean ± S.D., the urine, feces, expired air and cage wash samples in which the radioactivity was less than twice the background for the system or the radioactivity was less than 0.05% of the dose were considered to have a value of zero.

Pharmacokinetic parameters for ISIS 2105 equivalents in the blood and plasma were calculated by polyexponential curve fitting of the observed concentrations by using RSTRIP Polyexponential Curve Fitting Program, version 4.02 (Micromath Scientific Software, Salt Lake City, UT). Areas under the concentration-time curve and elimination half-lives for the tissues were calculated by using noncompartmental analysis of the observed data (Shumaker, 1986).

Results

Radioactivity Levels at the Site of Injection

Skin samples at the site of injection were analyzed to determine the extent of local exposure to the drug and the potential local metabolism of the drug. Figure 1 shows the percentage of a 3.7-mg/kg dose that remained at the site of injection as a function of time in two animals per time point and the average of the results from the two animals. Rapid loss from the injection site occurred with 65% of the dose being absorbed

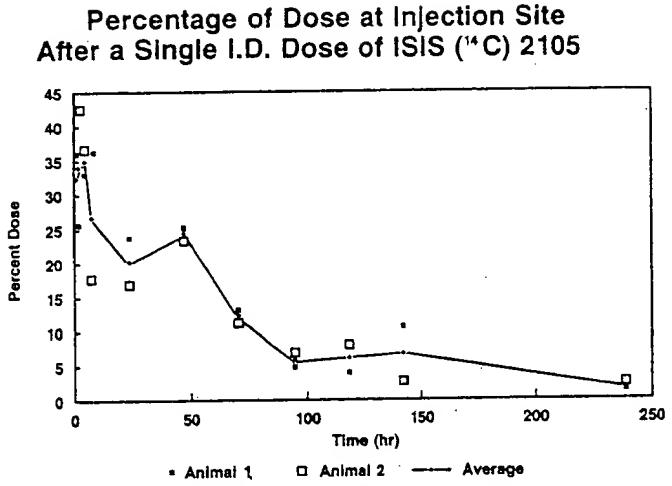

Fig. 1. Percentage of initial radioactivity remaining at the injection site after a single intradermal dose of approximately 3.7 mg/kg of [^{14}C]2105. ■ and □ are data from two individual animals; ♦ is the average.

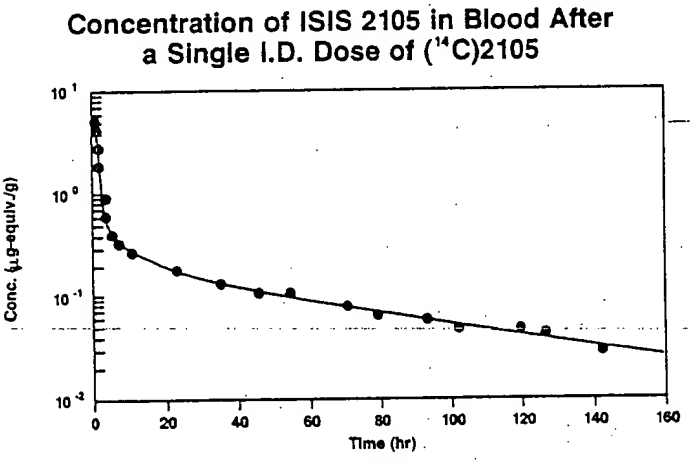

Fig. 2. Concentration of ISIS 2105 equivalents in the blood after a single intradermal dose of approximately 3.7 mg/kg of [^{14}]2105. Each point represents the average of the values for two animals. The broken line represents the best-fit estimates from a three-compartment model.

within 1 hr. However, the rapid initial phase was followed by a terminal local clearance phase of 56 hr.

Pharmacokinetic Properties of ISIS 2105 Equivalents in the Blood and Plasma

Essentially all the radioactivity in the blood was in the plasma and essentially all the plasma radioactivity was bound to serum albumin and α_2-macroglobulin, as we previously found after i.v. doses of [^{14}C]2105 (J. Leeds, unpublished data).

Figure 2 shows the concentration of [^{14}C]2105 equivalents in the blood as a function of time after a single intradermal dose. Absorption was rapid. Thirty minutes after a dose, the concentration of [^{14}C]2105 equivalents was 5 μg/g of blood. The blood radioactivity decay profile was triexponential, with a distribution phase of 0.5 hr, an initial elimination phase of 6.04 hr and a terminal elimination phase of 52.80 hr. The initial volume of distribution was 17.2 ml and the postdistributive volume of distribution was 2162 ml, indicative of extensive partitioning into the tissues.

Tissue Distribution and Pharmacokinetics

Table 1 shows the fraction of a single dose of [^{14}C]2105 found in various tissues at several times after intradermal injection.

TABLE 1

Percentage of dose in tissues[a] of female Sprague-Dawley rats at intervals after a single intradermal administration of [^{14}C]2105 at a dose of 3.7 mg/kg

Time	Fraction of Dose[b]					
	Liver	Renal Medulla[c]	Renal Cortex[c]	Skeletal Muscle[d]	Bone Marrow[e]	Skin
hr	%					
1	7.8	—[f]	2.8	2.1	5.7	32.3
2	11.6	—	4.4	1.3	5.9	34.2
4	12.6	—	5.0	1.3	5.4	34.7
8	11.5	1.0	4.4	—	5.1	26.6
24	11.5	1.0	8.1	—	7.7	20.0
48	7.8	1.0	5.3	—	6.9	24.0
72	8.9	1.0	8.9	—	8.4	11.9
96	5.9	—	5.8	—	7.2	5.4
120	3.6	—	5.2	—	6.9	5.6
144	2.0	—	4.0	—	4.0	6.2
240	1.5	—	3.3	—	2.7	1.3

[a] Tissues containing ≥1% dose are included. Lung, brain, spleen, ovaries, uterus and eyes contained <1% dose at any given time.

[b] All percentages are average data from two animals, except for time 240 (hr), which is mean data from four animals.

[c] Percentages were calculated by assuming that renal cortex = 69% and renal medulla = 31% of the total kidney weight (percentages generated from kidney dissections).

[d] Percentages were calculated from the organ weights and by assuming that muscle = 50% of the body weight (Burka *et al.*, 1987).

[e] Percentages were calculated by assuming that bone marrow = 3% of the total body weight (Baker *et al.*, 1979).

[f] Contained <1% dose.

Most of the radioactivity in the skin was at the injection site. Liver, renal cortex and bone marrow accumulated radioactivity rapidly and extensively.

Table 2 shows the pharmacokinetic parameters in the blood, with selected pharmacokinetic parameters in tissues that accumulated more than 1% of the dose (table 1), in expired air and in urine. The liver and renal medulla appeared to accumulate the drug relatively rapidly; the renal cortex and bone marrow reached maximum concentrations more slowly. The elimination half-lives varied from 58 hr in the liver to more than 150 hr in the renal cortex and bone marrow. The terminal half-lives, calculated from urine and air excretion, approximated those calculated from blood and plasma elimination curves.

Metabolism of [^{14}C]2105

Site of injection. Skin samples at the site of injection were extracted as described under Materials and Methods. The recovery of radioactivity was variable (15–60%), probably because of difficulty in achieving efficient tissue homogenization and in the digestion of connective tissue. However, the fraction of radioactivity represented by the intact drug did not vary in relationship to the extent of total radioactivity extracted. Table 3 shows a group of synthetic standards and their retention times on the HPLC system. Figure 3 shows the chromatograms obtained from skin samples at several times after dosing. Intact ISIS 2105 was present at the injection site at the 72-hr time point but, by 96 hr postinjection, all the radioactivity at the injection site was fully degraded.

Plasma and other tissues. Table 4 shows the percent of radioactivity extracted from the plasma and other tissues that comigrated with intact ISIS 2105 on anion-exchange HPLC. The recovery of total radioactivity from these tissues usually exceeded 60% and no variation in chromatographic pattern was observed as a function of the fraction of radioactivity extracted.

TABLE 2

Pharmacokinetic parameters of ISIS 2105 equivalents in blood and other tissues of rats after a single intradermal administration of [^{14}C]2105 at a dose of 3.7 mg/kg

Tissue	$AUC_{(0,\infty)}$[a]	Distribution $T_{½}$[b]	Intermediate $T_{½}$[b]	Terminal $T_{½}$[b]	C_{max}[c]	T_{max}	Apparent Clearance	V_{dss}[d]	V_c[e]
	μg-equivalents-hr/g	*hr*	*hr*	*hr*	*μg-equivalents/g*	*hr*	*ml/hr*	*ml*	*ml*
Blood	27	0.5	6	53	5.1	0.5	28.4	2162	17.2
Plasma	54			42	8.2	1			
Urine	—			72	—	—			
Expired air	—			53	—	—			
Skin (injection site)	—			56	—	—			
Liver	1006			58	11.4	4			
Renal medulla	2597			124	18.6	4			
Renal cortex	8507			156	61.0	72			
Bone marrow	1555			157	10.0	72			

[a] Area under the curve from zero to infinity.
[b] Half-life.
[c] Maximum concentration.
[d] Postdistribution volume of distribution.
[e] Initial volume of distribution.

TABLE 3

Retention times of standards on HPLC

Compound	Retention Time	Length	Sequence
	min		
2105	44.8	20 mer	5′-TTG-CTT-CCA-TCT-TCC-TCG-TC-3′
5554	43.7	18 mer	5′-GCT-TCC-ATC-TTC-CTC-GTC-3′
5555	41.1	16 mer	5′-TTC-CAT-CTT-CCT-CGT-C-3′
5556	39.0	14 mer	5′-CCA-TCT-TCC-TCG-TC-3′
5557	36.2	12 mer	5′-ATC-TTC-CTC-GTC-3′
5558	32.4	10 mer	5′-CTT-CCT-CGT-C-3′
5569	28.2	8 mer	5′-TCC-TCG-TC-3′
5571	22.9	6 mer	5′-CTC-GTC-3′
5564	43.6	18 mer	5′-TTG-CTT-CCA-TCT-TCC-TCG-3′
5506	40.6	16 mer	5′-TTG-CTT-CCA-TCT-TCC-T-3′
5565	38.1	14 mer	5′-TTG-CTT-CCA-TCT-TC-3′
5572	35.4	12 mer	5′-TTG-CTT-CCA-TCT-3′
5573	32.3	10 mer	5′-TTG-CTT-CCA-T-3′
5574	27.5	8 mer	5′-TTG-CTT-CC-3′
5570	22.4	6 mer	5′-TTG-CTT-3′
Thymidine	1.4		
Thymine	1.7		

However, the separation of intact ISIS 2105 from 18-mer compounds (table 3) is such that it is difficult to discriminate between the three species (intact ISIS 2105 and two possible 18-mer compounds) and, thus, the percentages shown in table 4 are estimates of the minimum fraction of radioactivity present in the ISIS 2105 peak. As can be seen, 1 hr after dosing, approximately 70% of the radioactivity in the plasma comigrated with intact ISIS 2105 and about 40% was intact at 8 hr. Minimal intact ISIS 2105 was detected at the 24-hr time point, mainly because of the insufficient counts (table 4).

Four hours after injection, approximately 80% of the radioactivity in the liver was intact ISIS 2105 but, by 48 hr, only 15% of the radioactivity was intact ISIS 2105. By contrast, only approximately 40% of the radioactivity in the kidney 4 hr after a dose was intact ISIS 2105 but there was minimal appreciable reduction in the percent of intact drug over the 72 hr during which samples were taken for analysis.

Elimination

Figure 4A shows the cumulative excretion of radiolabel in expired air in four animals that received 3.7 mg/kg of [^{14}C]2105 intradermally. A mean of 50.0 ± 2.9% of the dose was cleared in the expired air within 240 hr after the dose.

Figure 4B shows the cumulative excretion of radioactivity in urine in the same four animals. A mean of 17.2 ± 2.7% of the dose was cleared in the urine within 240 hr after the dose. HPLC analysis demonstrated that virtually none of the radioactivity in the urine at any time represented intact ISIS 2105 (data not shown).

Fecal elimination accounted for a mean of 5 ± 3.2% of the radioactivity administered after 240 hr.

At 240 hr after an intradermal dose, 14.5 ± 1.28% of the radioactivity remained in the tissues. The renal cortex (3.3 ± 0.78%), liver (1.5 ± 0.19%), bone marrow (2.8 ± 0.04%) and the injection site (1.3 ± 0.19%) were the tissues with the greatest levels of radioactivity. The total recovery from excretion and the carcass ranged from 82% to 92% of the initial dose (mean, 88.0 ± 4.3%).

Discussion

[^{14}C]2105 was rapidly absorbed after an intradermal dose of 3.7 mg/kg. Figure 1 demonstrates that approximately 65% of the dose was eliminated from the injection site within 1 hr after the dose and blood levels were achieved within 30 min. After the initial rapid phase of absorption, there was a much more protracted period in which radioactivity was eliminated from the injection site with a terminal half-life of 56 hr. Approximately 95% of the dose was cleared from the injection site by 240 hr.

Although ISIS 2105 was metabolized at the site of injection, intact ISIS 2105 was well absorbed. Extensive local metabolism was not evident before time points when more than 70% of the drug was absorbed. Moreover, 1 hr after the dose, 70% of the radioactivity in plasma was intact ISIS 2105.

The metabolic pattern observed at the injection site was similar to that observed in other tissues after i.v. administration of a similar (3.6 mg/kg) dose (Cossum *et al.*, 1993). The rate of metabolism observed in the skin was somewhat slower than that observed in the liver but it was similar. Based on *in vitro* studies, levels of intact drug would be greater than the IC_{90} for human papillomavirus for more than 3 to 4 days at the projected clinical dose. Thus, a twice-weekly or weekly schedule of administration is supported by the intradermal pharmacokinetic properties.

Once absorbed, the pharmacokinetic properties, distribution and metabolism of ISIS 2105 after intradermal administration

HPLC Analysis of Skin at Injection Site Following a Single I.D. Administration of (^{14}C) ISIS 2105

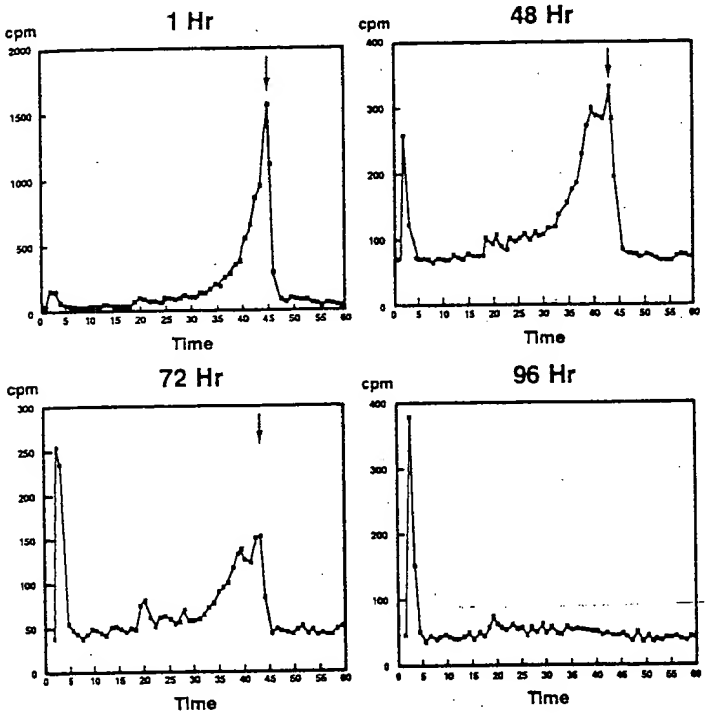

Fig. 3. Strong anion-exchange HPLC radiochromatograms of skin at injection site samples from Sprague-Dawley rats at various times after intradermal administration of approximately 3.7 mg/kg of [^{14}C]2105. The samples were prepared as described under Materials and Methods. Panels A, B, C and D represent samples taken 1, 48, 72 and 96 hr postdosing, respectively. The arrows indicate the elution time for the ISIS 2105 standard. An arrow identifies ISIS 2105.

were comparable to those after an i.v. dose (Cossum *et al.*, 1993). Table 5 compares several pharmacokinetic parameters derived from analyses of blood from animals dosed i.v. or intradermally. In our study of i.v. dose pharmacokinetic properties, the plasma clearance data were best accommodated by a quadriexponential equation. By contrast, in this study, the data were best accommodated by a triexponential equation. We do not believe this difference is biologically significant. The distribution and terminal elimination phases were entirely comparable. The volumes of the central compartment were comparable and the total volumes of distribution were large. The larger total volume of distribution in the intradermal study was probably caused by prolonged retention of a fraction of the radiolabel at the site of injection.

Excluding the local skin concentration at the site of injection, the tissue distribution of ISIS 2105 after intradermal administration was comparable to that observed after i.v. dosing. The liver, renal cortex and bone marrow accumulated the highest level of the drug. The liver appeared to accumulate the drug slightly more rapidly than did renal cortex and bone marrow and to have a shorter terminal half-life than the renal cortex and bone marrow.

The extent of and rate of metabolism of [^{14}C]2105 in the liver after intradermal administration was analogous to that observed after i.v. administration. Compared with i.v. dosing, in the kidney after intradermal dosing, more degradates were observed at earlier times after dosing. This may reflect the absorption of degradates from the skin and entrapment of these compounds in the kidney.

The elimination of [^{14}C]2105 occurs by slow metabolism and excretion, primarily in expired air, irrespective of the route of administration. In these studies, ISIS 2105 was labeled at the carbon-2 position of thymidine, a site that is metabolized to CO_2 (Cossum *et al.*, 1993). In both the i.v. and intradermal studies, approximately 50% of the dose was found in the expired air. Only approximately 15% and 5% were found in the urine and feces, respectively, and no intact ISIS 2105 was found at any time in the urine or feces.

In conclusion, in this study, we demonstrated that [^{14}C]2105 is rapidly and extensively absorbed after intradermal administration. Once absorbed, the drug behaves as if it were injected i.v. Slow metabolism occurs in the skin and other tissues and accounts for clearance of the drug. Based on probable local concentrations of intact ISIS 2105 and estimates of potency in inhibiting papillomaviruses (Cowsert *et al.*, 1993), once or twice a week or less frequent intralesional dosing in patients with genital warts would seem sensible.

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TABLE 4

Percent of extractable radioactivity comigrating with ISIS 2105 in anion-exchange HPLC

Time	Percent Intact ISIS 2105		
	Plasma	Liver	Kidney
hr		%	
1	70		
4	59	82	44
8	40		
24		20	33
48		15	
72			35
240			5.3

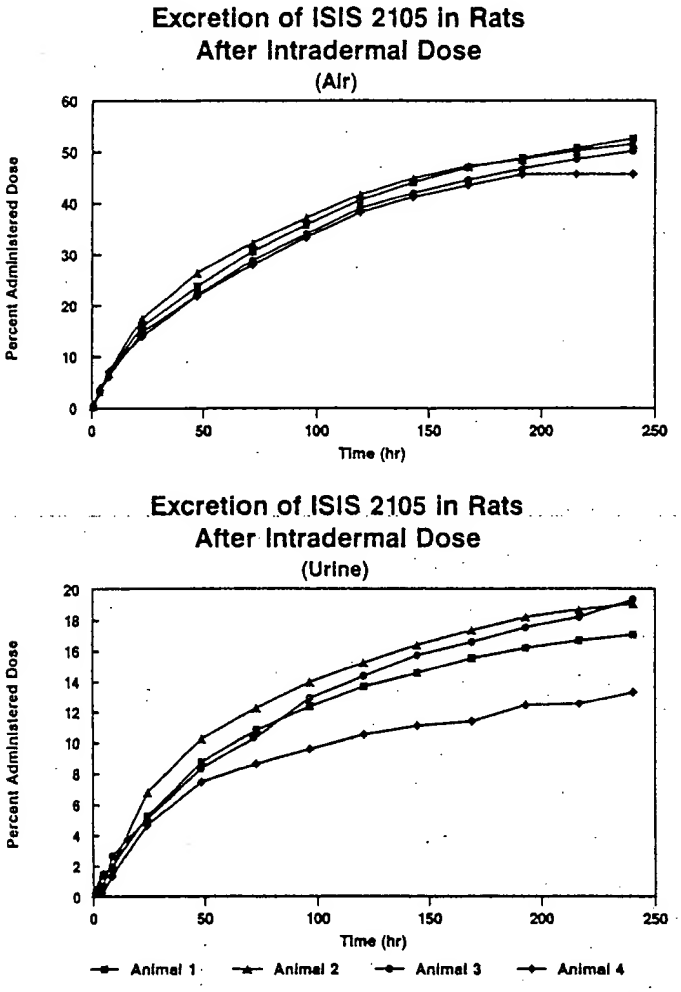

Fig. 4. A) The cumulative percent dose excreted in expired air after a single intradermal dose of [^{14}C]2105 of approximately 3.7 mg/kg. The data for individual animals are plotted. B) The cumulative percent dose excreted in the urine after a single intradermal dose of [^{14}C]2105 of approximately. 3.7 mg/kg. The data for individual animals are plotted.

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TABLE 5

Comparison of selected pharmacokinetic parameters after i.v. or intradermal doses of [^{14}C]2105

The pharmacokinetic parameters for intravenous dosing are taken from Cossum *et al.*, 1993.

Route	Parameter			
	Distribution $T_{1/2}$[a]	Terminal $T_{1/2}$[a]	V_c[b]	V_{dss}[c]
	hr	*hr*	*ml*	*ml*
Intravenous	0.4	51	22.0	1076
Intradermal	0.5	53	17.2	2162

[a] Half-life.
[b] Initial volume of distribution.
[c] Postdistribution volume of distribution.

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Medical Sciences

EXHIBIT 3

Pharmacokinetics, biodistribution, and stability of oligodeoxynucleotide phosphorothioates in mice

(antisense inhibition of gene expression/antiviral therapy/oligodeoxynucleotide uptake/human immunodeficiency virus)

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ABSTRACT **We describe preliminary studies of the pharmacokinetics, biodistribution, and excretion of an oligodeoxynucleotide phosphorothioate ([S]oligonucleotide) in mice. After either intravenous or intraperitoneal administration of a single dose (30 mg/kg of body weight), [S]oligonucleotide (^{35}S-labeled at each internucleotide linkage) was found in most of the tissues for up to 48 hr. About 30% of the dose was excreted in urine within 24 hr, irrespective of the mode of administration; the excreted [S]oligonucleotide was found to be extensively degraded. In plasma, stomach, heart, and intestine, the [S]oligonucleotide was degraded by only 15%, whereas in the kidney and liver degradation was about 50% in 48 hr. The surprising observation was made that chain length extension of administered [S]oligonucleotide occurred in kidney, liver, and intestine. These results provide an initial definition of parameters for the pharmaceutical development of antisense oligonucleotides.**

Previous studies demonstrated that replication of human immunodeficiency virus (HIV) could be inhibited by normal phosphodiester oligonucleotides complementary to HIV RNA (1–3). However, the relatively short half-life of normal oligonucleotides in serum and in cells, due to the presence of nucleases, limits their potential usefulness *in vivo* (4). To overcome this limitation, oligonucleotides with modified internucleoside phosphate backbones, such as methylphosphonates (5, 6), phosphorothioates, and several phosphoramidate analogues (3, 7–12), have been studied. These analogues of oligodeoxynucleotides are more resistant to nucleases than their unmodified counterparts, and they should therefore exhibit longer survival times *in vivo*. Oligodeoxynucleotide phosphorothioates ([S]oligonucleotides) are effective in inhibiting HIV replication in tissue culture, with ID_{50} values (inhibitory dose causing 50% suppression of virus) as low as 10–100 nM (3).

Our continued interest in developing antisense oligonucleotides as possible chemotherapeutic agents led us to investigate the pharmacokinetics, biodistribution, and half-life of [S]oligonucleotides in mice.

EXPERIMENTAL PROCEDURES

Synthesis of Oligodeoxynucleotides. [S]Oligonucleotides were synthesized on an automated synthesizer (model 8700; Milligen/Biosearch, Novato, CA) using H-phosphonate chemistry on controlled-pore glass, followed by oxidation with 0.2 M sulfur in carbon disulfide/pyridine/triethylamine (9:9:1, vol/vol). The syntheses were carried out on a 5- to 10-μmol scale. The [S]oligonucleotides were purified by low-pressure ion-exchange chromatography (DEAE-cellulose, DE-52; Whatman) followed by reverse-phase chromatography (C_{18}) and finally dialysis (10). The [S]oligonucleotide synthesized for the present study had the base sequence 5′-ACA-CCC-AAT-TCT-GAA-AAT-GG-3′, which is complementary to the HIV *tat* splice acceptor site (nucleotides 5349–5368).

^{35}S-Labeled [S]Oligonucleotide. Five milligrams of oligodeoxynucleoside H-phosphonate (20-mer, same sequence as above) bound to controlled-pore glass was oxidized with a mixture of $^{35}S_8$ (5 mCi, 1 Ci/mg, Amersham; 1 Ci = 37 GBq) in 40 μl of carbon disulfide/pyridine/triethylamine (9:9:1). After 30 min, 100 μl of 7% (wt/vol) unlabeled S_8 in the same solvent mixture was added and the reaction continued for another 60 min. The solution was removed and the support was washed with carbon disulfide (3 × 500 μl) and with acetonitrile (3 × 700 μl). The product was deprotected in concentrated ammonia (55°C, 14 hr), evaporated, and desalted with a Sep-Pak C_{18} column (Waters). The resultant product was purified by 20% polyacrylamide gel/7 M urea electrophoresis. The desired band was excised under UV illumination and the [S]oligonucleotide was extracted from the gel and desalted. The yield was five A_{260} units (150 μg; specific activity, 5 × 10^9 cpm/μmol; 0.44 μCi/μg). Purified ^{35}S-labeled [S]oligonucleotide 20-mer contained ≈2% 19-mer.

Mice. Male CD2F1 mice (average weight, 20 g) were used. The [S]oligonucleotide was dissolved in sterile water. The concentration of the solution was adjusted so that 200 μl administered to a 20-g mouse resulted in a dose of 30 mg/kg of body weight (specific activity, 8.02 μCi/mg). ^{35}S-labeled [S]oligonucleotide was mixed with unlabeled [S]oligonucleotide to obtain the desired concentration.

The dose was administered intravenously via the tail vein in one group of mice and intraperitoneally in another group. At 5 min, 15 min, 30 min, 1 hr, 2 hr, 3 hr, 6 hr, 9 hr, 12 hr, 24 hr, and 48 hr following intravenous administration, one test animal was sacrificed and blood and organs were removed. At 1, 2, 3, 6, 9, 12, 24, and 48 hr following intraperitoneal administration one test animal was sacrificed and blood and organs were removed for determination of total radioactivity. The organs were homogenized (in deionized water, 1:10 dilution) prior to the determination of total radioactivity. The homogenates were kept at −20°C until extractions.

The urine and feces were collected in metabolism cages for total radioactivity determination from each of three animals following both intravenous and intraperitoneal routes of administration. Samples were collected from 0 to 6 hr, 6 to 12 hr, and 12 to 24 hr.

Extractions of [S]Oligonucleotides from Tissue Homogenates. Homogenized tissue (200 μl) was treated with proteinase K (2 mg/ml) in extraction buffer (0.5% SDS/10 mM

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Abbreviations: HIV, human immunodeficiency virus; [S]oligonucleotide, oligodeoxynucleotide phosphorothioate.

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NaCl/20 mM Tris·HCl, pH 7.6/10 mM EDTA) for 2 hr at 37°C. The samples were then extracted twice with phenol/chloroform (1:1, vol/vol) and once with chloroform. After ethanol precipitation, the oligonucleotides were analyzed by electrophoresis in 20% polyacrylamide gels containing 7 M urea. The gels were fixed in 10% acetic acid/10% methanol solution before autoradiography.

RESULTS

Excretion and Status of [S]Oligonucleotide in Urine. Following a dose of 30 mg/kg given either intravenously or intraperitoneally, ≈30% of the administered dose was excreted in the urine over 24 hr (Table 1). In a separate experiment, three mice were injected intravenously with [S]oligonucleotide as in Table 1 and 36% was again found to be excreted in the urine over the first 24 hr. From 24 to 48 hr, urine was collected by catheterization, which revealed an additional 8.7% excretion (average) of [S]oligonucleotide (data not shown). The excretion in urine was found to be independent of the route of administration. The dose excreted in feces was <1% in the first 24 hr, followed by 15% between 24 and 48 hr (data not shown).

The urine was analyzed by gel electrophoresis to check the status of the [S]oligonucleotide. For the intravenous route of administration, there was no degradation of [S]oligonucleotide in urine collected between 0 and 6 hr; about 5% degradation was observed in 6–12 hr and 15% in 12–24 hr. In contrast, for the intraperitoneal route, 75% of the [S]oligonucleotide in urine collected between 0 and 6 hr was degraded and by 12–24 hr degradation was about 90% (Fig. 1).

Bioavailability and Status of [S]Oligonucleotide in Plasma and Tissues. Plasma levels of [S]oligonucleotide in mice were rapidly depleted after either intravenous or intraperitoneal administration with a concomitant accumulation of oligonucleotide in the tissues (Figs. 2 and 3). As expected, higher plasma concentrations were found after intravenous administration than after intraperitoneal administration.

[S]Oligonucleotide concentration in the kidney and liver was markedly higher than in other tissues after either route of administration. The [S]oligonucleotide accumulated with time in these two organs up to 48 hr, the end point of the present study. In the other tissues studied there was an elimination of the [S]oligonucleotide with time from the initial higher concentration. The highest concentrations were found in the kidney. The [S]oligonucleotide did not appear in the brain in significant amounts.

Gel electrophoresis of [S]oligonucleotide recovered from plasma indicated that the circulating material remained quite intact over 24 hr (Fig. 4), although obviously decreasing in total amount (see Fig. 3). The same overall result was obtained with [S]oligonucleotide recovered from small and large intestine (Fig. 5). In kidney (Fig. 6) and liver (data not shown), a much larger fraction of the [S]oligonucleotide recovered at each time point was present as degraded fragments, approaching 30–40% of the total [S]oligonucleotide at 24 hr and 50% at 48 hr (Fig. 7).

Table 1. Urinary excretion of [S]oligonucleotide

Mouse	% dose recovered in urine			
	0–6 hr	6–12 hr	12–24 hr	Total
Intravenous administration (30 mg/kg)				
IV1	18.2	4.39	7.51	30.1
IV2	6.76	16.4	9.64	32.8
IV3	28.2	4.65	9.44	42.3
Intraperitoneal administration (30 mg/kg)				
IP1	9.11	2.23	17.6	28.9
IP2	6.35	8.09	12.6	27.0
IP3	10.1	7.35	13.1	30.6

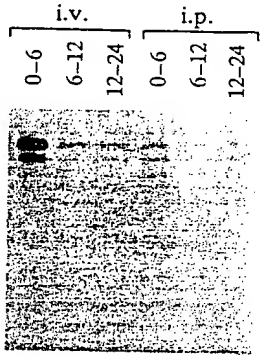

FIG. 1. Polyacrylamide gel electrophoresis of ^{35}S-labeled [S]oligonucleotide in urine excreted from mice receiving a dose of 30 mg/kg administered intravenously (i.v.; mouse IV3) or intraperitoneally (i.p.; mouse IP3). The urine was collected during periods 0–6 hr, 6–12 hr, and 12–24 hr after injection.

The gel electrophoretic analysis of the [S]oligonucleotide recovered from kidney displayed the progressive appearance at 6 hr of two bands of slower mobility than the initially administered oligonucleotide (Fig. 6). From the mobility of these two bands it appears that they might represent the addition of nucleotides to the [S]oligonucleotide. At 24 hr, ≈10% of the [S]oligonucleotide was in this form.

In the case of intestine, a more pronounced alteration of [S]oligonucleotide electrophoretic mobility was observed. A

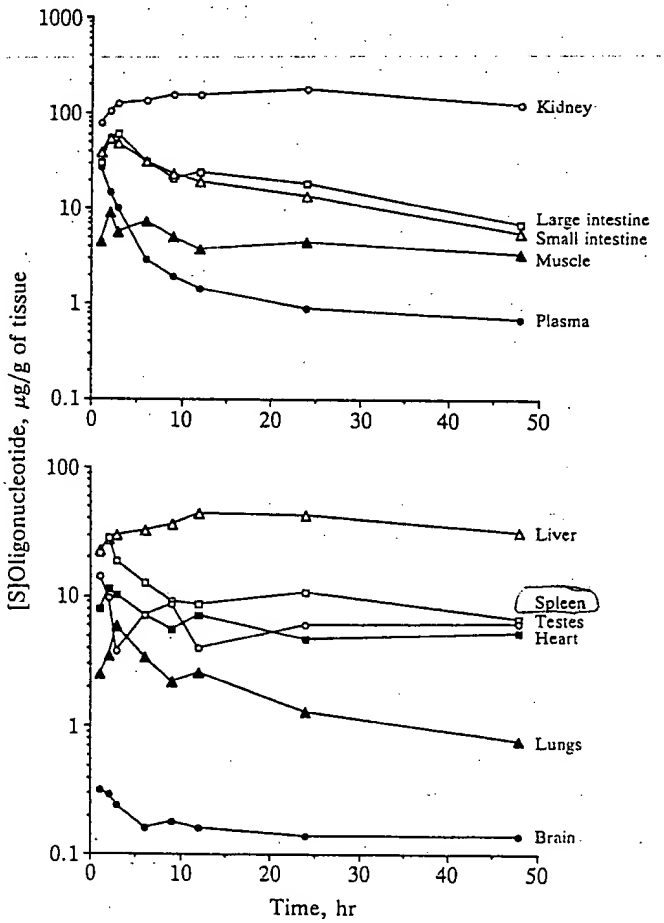

FIG. 2. Tissue levels of [S]oligonucleotide in mice after 30 mg/kg was administered intraperitoneally. One mouse was killed at each time point and the total radioactivity was determined in various organs. The tissue concentrations of oligonucleotide presented here are based on the radioactivity determination.

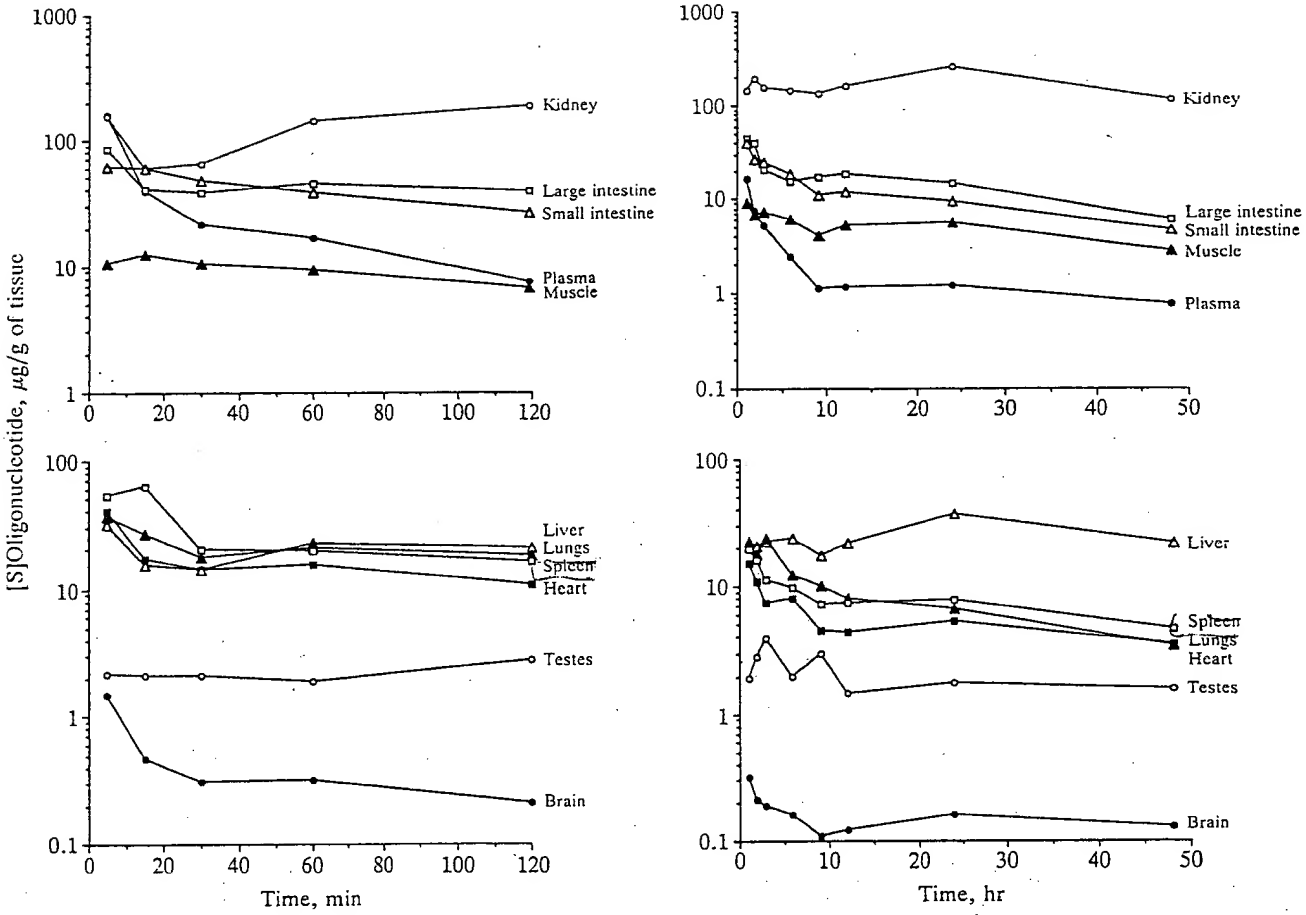

FIG. 3. Tissue levels of [S]oligonucleotide in mice 5–120 min (*Left*) or 1–48 hr (*Right*) after 30 mg/kg was administered intravenously. The concentrations of oligonucleotide in tissues were calculated from the total ^{35}S radioactivity.

portion of the ^{35}S radioactivity migrated much slower than the injected [S]oligonucleotide (Fig. 5). In the case of large intestine (Fig. 5 *Upper*), at only 5 min postinjection there was a series of bands that migrated behind the initial [S]oligonucleotide. These bands appeared to represent an oligomeric series. There were also some very high molecular weight bands that barely entered the gel (see 30-min, 1-hr, 3-hr, and 9-hr lanes). In small intestine (Fig. 5 *Lower*) these high molecular weight bands were more evident, whereas bands of intermediate mobility were less apparent. In both large and small intestine, there was no evidence of bands migrating immediately behind the initial oligonucleotide as was observed in kidney (Fig. 6).

DISCUSSION

[S]Oligonucleotides at concentrations of 10–100 nM are effective in inhibiting HIV replication in cell culture (3). The inhibition of HIV replication in chronically infected cells is sequence-dependent, and only complementary [S]oligonucleotides are effective (9, 10). A preliminary toxicity study of [S]oligonucleotides in mice and rats has shown that a dose of 150 mg/kg of body weight is nontoxic if given intraperitoneally (3). Daily administration, either intraperitoneally or subcutaneously of [S]oligonucleotide at 100 mg/kg of body weight for 14 days causes no observable toxicity or mortality in mice (unpublished data).

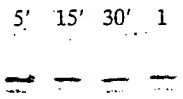

FIG. 4. Stability of [S]oligonucleotide in plasma after intravenous administration to mice. The ^{35}S-labeled [S]oligonucleotide injected was a 20-mer with a trace of 19-mer. At times indicated above the lanes [5 min (5′) to 24 hr], the mice were sacrificed and the oligonucleotide was extracted from plasma and analyzed by gel electrophoresis followed by autoradiography.

The present study shows that [S]oligonucleotide administered either intravenously or intraperitoneally to mice are initially distributed in most of the tissues and then are depleted. About 30% of the [S]oligonucleotide was excreted in urine after 24 hr regardless of the route of administration or the level of [S]oligonucleotide in plasma. Less than 1% of the [S]oligonucleotide was excreted in feces during the first 24 hr, and an additional average 15% was excreted between 24 and 48 hr. In a parallel study, an oligonucleotide (20-mer) containing 15 phosphodiester linkages and 4 phosphorothioate linkages (contiguous at the 5′ end) was excreted in urine to about 75% of the administered dose (30 mg/kg) in 12 hr (data not shown).

The [S]oligonucleotide was quite stable in most of the tissues except kidney and liver. Only 10–15% degradation was observed. The tissues from which [S]oligonucleotide was extracted and analyzed by gel electrophoresis included plasma, heart, spleen, large and small intestine, and stomach. In kidney and liver, [S]oligonucleotide was degraded with time, most probably by exonucleases (as suggested from the pattern of degradation). The rate of degradation was found to be almost linear with time, and about 50% was degraded in 48 hr.

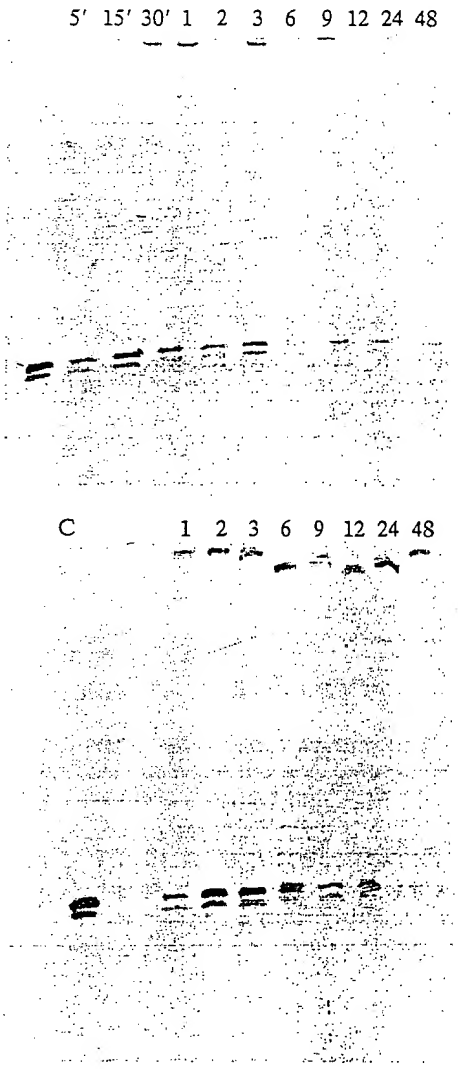

FIG. 5. Status of the ^{35}S-labeled [S]oligonucleotide (20-mer) in large intestine after intravenous administration (*Upper*) and in small intestine after intraperitoneal administration (*Lower*). Mice were killed at the times indicated [5 min (5') to 48 hr], and intestine was removed, homogenized, and extracted. Lane C shows the [S]oligonucleotide that was administered. Bands moving more slowly than the 20-mer were observed within 5 min.

In kidney and liver, new bands appeared on gel electrophoresis at the 1-hr time point, moving more slowly than the 20-mer band (Fig. 6). The intensity of this band increased

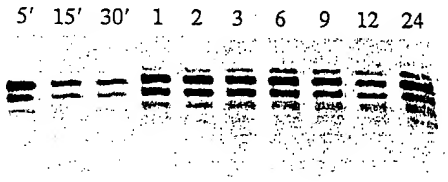

FIG. 6. Stability of [S]oligonucleotide in kidney after intravenous administration to mice. Mice were killed at the times indicated [5 min (5') to 24 hr], and kidneys were removed, homogenized, and extracted. Degradation was observed within 5 min and increased with time. By 24 hr, about 50% of the [S]oligonucleotide was degraded. Bands moving more slowly than the 20-mer could be observed by 1 hr. Similar results were obtained when kidney samples were from mice receiving [S]oligonucleotide intraperitoneally.

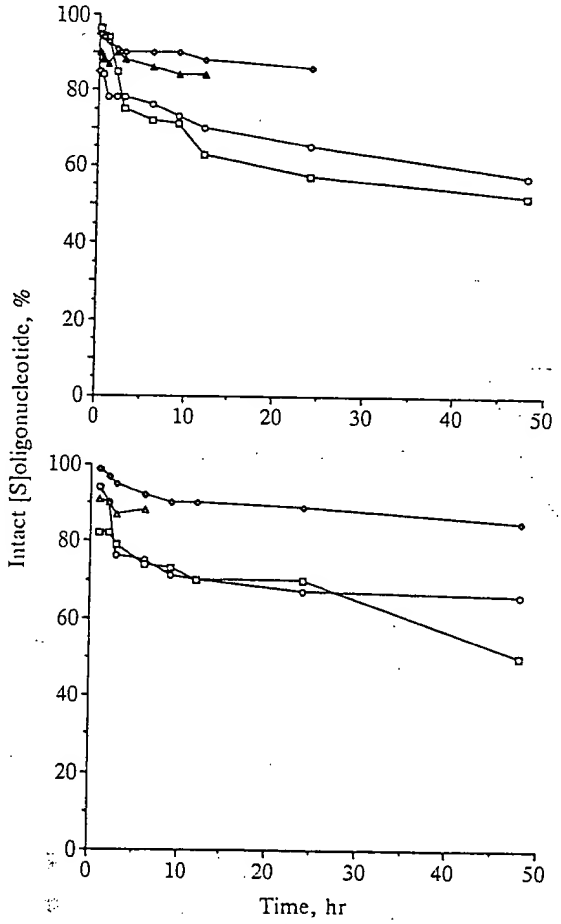

FIG. 7. Percentage of intact [S]oligonucleotide (20-mer) in liver (○), kidney (□), plasma (▲), and stomach (◆) after intravenous (*Upper*) and intraperitoneal (*Lower*) administration. The percentage is based on measurement of total radioactivity in tissues, followed by gel electrophoresis and autoradiography. The autoradiograph was scanned by a densitometer. In plasma, exact percentage of intact oligonucleotide at all time points could not be measured because of low radioactivity.

with time after injection, and by 6 hr an additional band appeared. By 24 hr, three bands moving more slowly than the administered 20-mer were observed. The mobilities of these additional bands are suggestive of the presence of extra nucleotides. A similar phenomenon was observed with [S]oligonucleotide recovered from liver (data not shown). The presence of the slower-mobility bands in both kidney and liver was observed with both intraperitoneal and intravenous routes of administration.

In the case of oligonucleotide extracted from large and small intestine, following either intravenous or intraperitoneal administration, a series of bands moving more slowly than 20-mer was observed (Fig. 5). It is possible that [S]oligonucleotide was being degraded and that nucleoside [[^{35}S]thio]phosphate was being incorporated into endogenous nucleic acids. However, it is not evident that this would lead to the series of discrete bands observed. A more likely explanation is ligation and/or extension of the oligonucleotide. Also noted in both small and large intestine was the presence of high molecular weight bands (Fig. 5). These may represent ^{35}S reincorporation, a limit extension product, or, conceivably, ligation of the [S]oligonucleotide to an endogenous macromolecule.

In summary, a pharmacokinetic study of [S]oligonucleotide in mice has demonstrated that the [S]oligonucleotide is distributed in most of the tissues and that up to 30% is excreted in urine in 24 hr and an additional 10% in 24–48 hr.

The oligonucleotide analogue appears to be degraded mostly by exonucleases. In some organs, such as kidney, liver, and intestine, it is extended in length.

We are indebted to Paul C. Zamecnik for encouragement, continuous guidance, and support in this work. We are also indebted to Dr. Thoru Pederson for critical review of the manuscript and comments. We gratefully acknowledge the advice and cooperation of Dr. Wayne Galbraith (APD Co., Alexandria, VA). The assistance of Dr. Salab El Dareer and Kathy Tillery of the Southern Research Institute (Birmingham, AL) in evaluating the tissue oligonucleotide concentrations is gratefully acknowledged. We thank Ms. Rebecca Bell and Mrs. Carol Tuttle for processing the manuscript and for expert secretarial assistance. This work was supported in part by a grant from the National Institute of Allergy and Infectious Diseases (U01 124846) and financial assistance from Hybridon, Inc.

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EXHIBIT 4

Chapter 5

In Vivo Pharmacokinetics of Oligonucleotides Following Administration by Different Routes

Valentin V. Vlassov, Leonid A. Yakubov, Valery Karamyshev, Ludmila Pautova, Elena Rykova, and Marina Nechaeva

CONTENTS

I. INTRODUCTION

Exciting results of studies on suppression of virus multiplication and attenuation of expression of deleterious genes with oligonucleotide derivatives[1-3] suggest that the compounds may become efficient therapeutics. For future therapeutic purposes, pharmacokinetic investigation of oligonucleotide behavior *in vivo* is needed for elucidation of optimal administration routes and estimation of therapeutic doses and frequency of administration of the compounds.

Studying on the pharmacokinetics of oligonucleotides just have begun, and most of the experiments have been done with phosphodiester oligonucleotides and phosphorothioate oligonucleotide analogs which are already being used in animal and human trials. These compounds behave similarly in animal organisms. A different behavior can be expected for the heavily modified and nonionic analogs of oligonucleotides; however, detailed pharmacological studies with these compounds have yet to be reported.

This chapter summarizes data on the *in vivo* pharmacokinetics of deoxyribooligonucleotides introduced by traditional intravenous and intraperitoneal injection and

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REJECTED

results of our recent studies on delivery of oligonucleotides in animals by various nondamaging (noninvasive) routes.

II. FACTORS AFFECTING DISTRIBUTION OF OLIGONUCLEOTIDES IN ORGANISMS

Distribution and fate of oligonucleotides in whole organisms are affected by a few factors. Oligonucleotides are relatively large and complex molecules and may be expected to interact with some macromolecules present in the bloodstream and at the cell surface. These interactions will affect distribution of the compounds in the organism, decrease bioavailability, and can cause toxic and other effects. It should be noted that oligonucleotides are not unknown foreign molecules to the organism, because the appearance of nucleic acids in the circulation can be caused by many events. They are detected in healthy patients and can appear in large amounts as a result of pathological disease (e.g., systemic lupus erythematosus), apoptosis, after trauma, and during hemodialysis. Studies in experimental animals have shown that DNA is removed rapidly and efficiently from circulation by liver.[4]

A. CELLULAR UPTAKE

The data on the *in vitro* pharmacokinetics of oligonucleotides and various oligonucleotide analogs were reviewed recently,[5,6] and more information concerning the interaction of oligonucleotides with cells can be found in other chapters of this volume. In this section we consider briefly the main features of the oligonucleotide-cell interaction, which can affect distribition of the compounds in organism and which should be taken into account when developing approaches for administration of the compounds.

In tissue culture experiments, it was found that eucaryotic cells take up deoxyribooligonucleotides by a mechanism compatible with receptor-mediated endocytosis.[5-7] The uptake process is saturable, specific, and dependent on temperature and concentration of the compounds. Plateau binding level is achieved by 1 to 2 h of incubation of cells with oligonucleotides at 37°C. Known inhibitors of endocytosis reduce uptake.

It was found that the cells can release the oligonucleotides taken up.[8] Efflux of the compounds occurs with a rate similar to that of the binding process. This phenomenon suggests that the occurrence of transcytosis and transportation of oligonucleotides through cell layers in tissues is a possibility.

In affinity labeling experiments and by affinity chromatography, 75 to 80 kDa cellular surface proteins capable of specific binding oligonucleotides were detected at the surface of various cells.[8-11] These proteins were suggested to be receptors specific for nucleic acids and are believed to play an important role in oligonucleotide internalization. The number of these putative receptors was calculated to be approximately 10^5 per one L929 cell. Also, a 35-kDa protein on the lymphocyte membrane was found to bind oligonucleotides, although interaction with this protein occurs only at acidic pH.[12] Recently we have found that phosphodiester oligonucleotides and phosphorothioate oligonucleotide analogs can bind to cellular CD4 receptors.[13] Interaction with cell-surface proteins can play an important role in the distribution of the compounds in organisms. Thus, the number of the 75- to 80-kDa proteins at the cells of different organs of mouse correlate with the quantity of the labeled oligonucleotide internalized by the organs.[14]

Phosphorothioate oligonucleotides enter cells in a manner similar to the phosphodiester oligonucleotides; however, they bind more tightly to the cellular receptors, and the uptake process occurs somewhat more slowly.[5,9]

Methylphosphonate analogs of oligonucleotides also were suggested to be taken up by cells by absorptive and fluid phase endocytosis.[15]

Numerous modifications were introduced in oligonucleotides for increasing their affinity to cell membranes and targeting to specific cells, taking advantage of receptor-mediated endocytosis.[3] Thus, conjugation of cholesterol residues to oligonucleotides increased efficiency of uptake of the compounds by cells in culture by more than an order of magnitude.[16] Conjugation to polycations[17] and absorption at the positively charged carriers[18] considerably increased the cellular uptake and allowed delivery of the compounds in the cell cytoplasm rather than in the endosomes. Apparently, the mentioned modifications of oligonucleotides and use of delivery vehicles will affect the fate of oligonucleotides in the organism.

B. INTERACTION OF OLIGONUCLEOTIDES WITH BLOOD PROTEINS

To investigate molecular interactions of oligonucleotides in the bloodstream, we have performed affinity labeling experiments with alkylating derivatives of oligonucleotides bearing an aromatic 2-chloroethylamino group capable of crosslinking to nucleic acids and proteins. The group was conjugated to the terminal phosphate, which is the least important position for interactions of oligonucleotides with macromolecules. We incubated the whole human blood serum with the [^{32}P]-labeled alkylating derivative of $(pT)_{16}$, and analyzed proteins reacted with the oligonucleotides. We have found that immunoglobulins M and G (IgM, IgG) and serum albumin are the major oligonucleotide-binding proteins in the blood. The reactivity decreased in the order: IgM>IgG>albumin; corresponding dissociation constants were estimated to be 4, 6, and 20 μM for the mentioned proteins, respectively.[19] Analysis of the labeled Ig revealed that the oligonucleotide derivatives react with both heavy and light chains of the proteins at, or in close proximity to, the antigen binding site. The interaction of oligonucleotides with the proteins should be taken into account when considering the fate and interactions of oligonucleotides in an organism, because the complexes can form in the organism at therapeutic concentrations of oligonucleotides *in vivo*. It also raises the question of other potential biological effects which may be caused by this interaction.

Specific immune response also can be a factor affecting the fate of oligonucleotides in an organism, although normal nucleic acids are known to be poor immunogens.[28] However, some oligonucleotide derivatives can form conjugates with blood proteins which can cause an immune response, as in the case of repeating administration of the reactive derivatives and affect biodistribution of the compounds. Thus, alkylating derivatives of oligonucleotides react with serum albumin and yield immunogenic conjugates.[20]

As expected, conjugation of oligonucleotides to groups facilitating cellular uptake of the compounds considerably affects the fate of the oligonucleotides in whole organism. Experiments with the cholesterol-conjugated oligonucleotides have shown that the compounds bind to low-density lipoproteins in the bloodstream, and this results in longer circulation and survival time of the compounds.[21]

III. PHARMACOKINETICS OF OLIGONUCLEOTIDES

A. ADMINISTRATION BY INTRAVENOUS, INTRAPERITONEAL, AND SUBCUTANEOUS ROUTES

1. Accumulation and Degradation of Oligonucleotides in Organs

Pharmacokinetics, biodistribution, and excretion of oligodeoxynucleotides and oligodeoxynucleotide phosphorothioates were investigated in experiments with different animals.[7,22-25] We investigated the fate in mice of the 5′-[^{32}P]-labeled oligonucleotides, bearing a benzylamine residue at the 5′-phosphate, for protection from enzymatic dephosphorylation. It was found that after intravenous administration, phosphodiester oligonucleotides rapidly distribute in mice and reach most of the animal organs.[7,24] In the case of intraperitoneal (i.p.) injection, the compounds appeared in the bloodstream after a

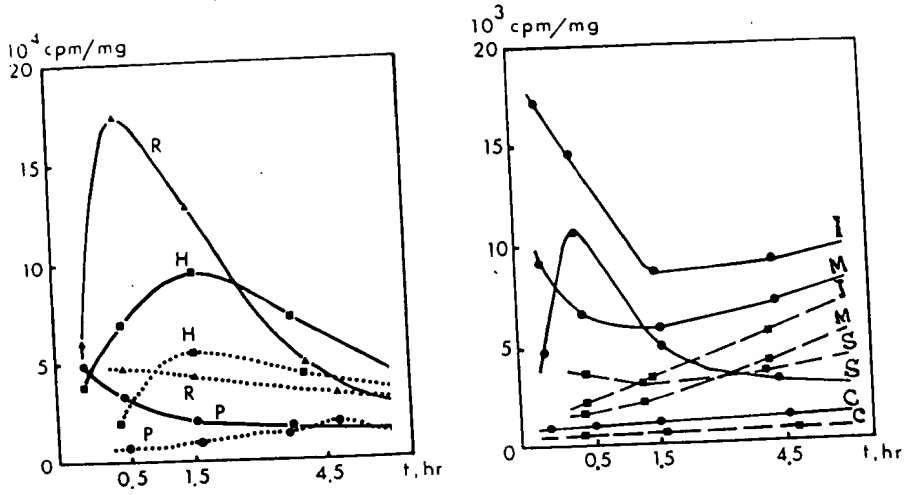

Figure 1 Kinetics of oligonucleotide accumulation in mice organs after the intraperitoneal and subcutaneous (dashed and dotted lines) administration. 2 nmol of the [^{32}P]-5'benzylphosphoramide derivative of the oligonucleotide pTGACCCTCTTCCCATC (2.5 Ci/mmol) was administered. After certain time points the mice were decapitated, and the radioactivity of blood and sample organs was determined in a liquid scintillation counter. S, blood; R, kidney; I, spleen; H, liver; P, pancreas; M, muscles; C, brain.

delay of about 30 min and tissue distributions were similar to the i.v. route (Figure 1a,b). Subcutaneously injected oligonucleotides distributed in an organism considerably slower. Accumulation of the label was more efficient in excreting organs and reticuloendothelial tissue in liver, spleen, and kidneys.[24] The brain was the least accessible for the compounds. When administered subcutaneously the compound concentrated more in the spleen and muscles.

A significant amount of the deoxyribooligonucleotide and degradation products was excreted with urine: 30 and 50% at 4 and 24 h postinjection, respectively. Similar results were obtained with phosphorothioate oligonucleotide derivatives.[22] Within the first 24 h, about 30% of the phosphorothioate oligonucleotides was excreted with urine and an additional 15% was excreted by 48 h. The excreted material was represented by intact and partially degraded oligonucleotides. Electrophoretic analysis of deoxyribooligonucleotides extracted from mice tissues after i.p. injection showed that half-life times of oligonucleotides vary in organs, from 15 min or less in liver to 30 min in blood and pancreas.

Phosphorothioate oligonucleotide analogs were much more stable in mice. By 30 min post-i.p. injection, the derivatives were practically undegraded in blood and pancreas; in most other organs the degradation rate was also diminished by a factor of 1.5 to 2, as compared to the phosphodiester oligonucleotides. By 48 h, degradation of phosphorothioate oligonucleotides in tissues varied from 50% in liver and kidney to 15% in plasma, stomach, intestine, and heart. The degradation pattern was characteristic of the 3'-exonuclease digestion. Surprisingly, oligonucleotides were extended in some organs such as liver, kidney, and intestine.[22] Accumulation of phosphorothioate oligonucleotides in the blood plasma of different animals (rat, rabbit, monkey) after single intravenous and intraperitoneal injections of the compound has been investigated.[25] It was found that peak plasma concentration was achieved a few minutes after the intravenous injection. Intravenously administered phosphorothioate oligonucleotide at doses of 5 mg/kg and 50 mg/kg resulted in the rat plasma concentrations of the compounds of 3 μM and 20 to 60

μM, respectively. The half life time of the compounds was approximately 70 h for the 50-mg/kg dose.[23,25] When the compounds were administered intraperitoneally, the plasma concentration-time curve was very similar to that for the intravenous administration, although the peak concentration was achieved 30 min later and the peak was lower. In accordance with the experiments with mice, it was found that in rabbit and monkey organisms oligonucleotides accumulate most intensively in the kidney and liver, and the lowest concentration was detected in the brain. In monkey, low accumulation of phosphorothioate compounds was observed in brain, cartilage, thyroid, and prostate. Moderate accumulation was detected in muscle, tongue, bladder, esophagus, duodenum, fat, gallbladder, and trachea. A high accumulation was characteristic of thymus, kidney, lymph nodes, liver, adrenal gland, lung, aorta, pancreas, bone marrow, heart, and salivary gland. The efficiency of accumulation of oligonucleotides in various tissues was highly correlated with the binding of oligonucleotides to membranes of the corresponding cells.[24,25]

In rats, up to 70% of the material present in plasma 20 h after intraperitoneal injection was represented by intact oligonucleotide, while in rabbits oligonucleotides survived for a longer time than in rats; degradation in monkeys was more rapid. It was concluded that daily injections are frequent enough to maintain therapeutic concentrations of phosphorothioate oligonucleotides in plasma.

2. Effect of Modifications and Delivering Devices on Oligonucleotide Pharmacokinetics

To elucidate the role of some oligonucleotide modifications on their fate in an organism, we injected the animals with [^{32}P]-labeled derivatives of phosphodiester oligonucleotides carrying an aromatic 2-chloroethylamino group, a phenazinium group, and a cholesterol group. We also investigated the fate of oligonucleotides with alternating phosphodiester and methylphosphonate linkages. The distribution of the compounds among animal organs was similar; however, a threefold greater amount of the last two compounds was bound to blood cells, as compared to the first one.[7] Electrophoretic analysis, performed 1 h postinjection, has revealed that the modifications provided a significant protection of the oligonucleotide moiety from degradation. To investigate the effect of incapsulation of the oligonucleotide derivatives in membrane carriers on their distribution in the organism, oligonucleotides were incorporated into multilamellar liposomes and into Sendai virus envelopes.[7] When the preparations were injected in mice, an enhanced delivery of the labeled oligonucleotides into lymphatic nodi and spleen was observed.

The pharmacokinetics of methylphosphonate oligonucleotide analog injected into the tail vein of a mouse has been investigated.[26] Within a few minutes the compound distributed among all animal tissues, with the lowest level found in the brain. Elimination of the compound from the circulation was rapid (half-life was 17 min). Within 2 h postinjection, up to 70% of the total amount of the oligomer injected was excreted with urine. It should be noted that the studies were performed with a chimeric oligonucleotide with a single phosphodiester linkage at the 5′ end. One can expect that the behavior of the charged oligonucleotide and electroneutral methylphosphonate analogs may be different.

B. PHARMACOKINETICS OF OLIGONUCLEOTIDES INTRODUCED BY NONDAMAGING ROUTES

1. Comparative Efficiency of Oligonucleotide Delivery by Different Administration Routes

To optimize therapy, it is essential to search for nondamaging routes of administration which would allow delivery of the compounds in a controlled release fashion. Oligonucleotides could be expected to be poorly absorbed by the oral route due to degradation in the gastrointestinal tract. Because of their polyanionic nature, they are hardly expected

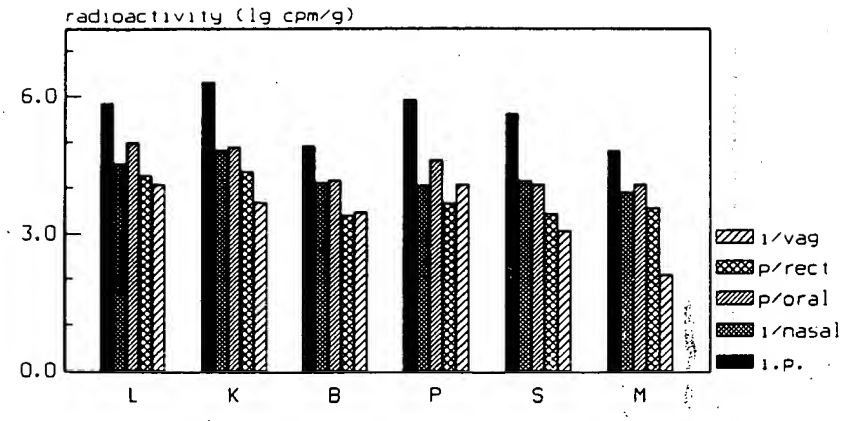

Figure 2 Distribution of oligonucleotides in mice organism after administration by different routes. 2 nmol of the [^{32}P]-5′benzylphosphoramide derivative of the oligonucleotide pTGACCCTCTTCCCATC (2.5 Ci/mmol) was administered. The mice were decapitated 20 min after the administration and tissue samples were weighed and counted. L, liver sample; K, kidney sample; B, blood sample; P, pancreas sample; S, spleen sample; M, muscle sample. I.p., intraperitoneal injection; i/nasal, intranasal injection; p/oral, per os injection; i/vag, oligonucleotide was introduced into the vagina of mice; p/rect, oligonucleotide was introduced into the rectum of mice. (From Vlassov, V.V., Karamyshev, V.N., and Yakubov, L.A., FEBS Lett., 327, 271, 1993. With permission.)

to penetrate through mucosa or nondamaged skin. However, keeping in mind the possibility of transcytosis and in hope for the existence of natural transport mechanisms, we investigated systematically the various routes of administration of phosphodiester oligonucleotides.

The experiments were performed with the 5′-^{32}P-labeled oligodeoxynucleotides The 5′-phosphate of the oligonucleotide was protected from dephosphorylation by conjugation to benzylamine. Equal amounts (2 nmol, 2×10^7 cpm) of the ^{32}P-labeled oligonucleotide derivatives were introduced in Balb mice. The compounds were dissolved in physiological salt solution and introduced intraperitoneally, intranasally (dropwise into the nose holes, total volume 5 µl), per os (dropwise into the mouth of mice, 50 µl), intravaginally (by Gilson® pipette into the vagina of mice, 10 µl), and rectally (in the distal part of the rectum, 10 µl). Application onto skin was performed as follows: 10 µl of the oligonucleotide solution was applied onto the skin of ear helices of mice. Special attention was paid to preventing the mice from licking the applied solution. In the case of ocular delivery, oligonuclotides were introduced in 2 ul of aqueous solution in mouse eye with a pipette.

Typically, mice were decapitated 20 min after the administration of oligonucleotides. Radioactivity of different organs was determined, and radioactive material extracted from the organs was subjected to analysis by electrophoresis. Figure 2 shows distribution of the label among mice organs after administration of the oligonucleotide derivative by different methods. Intranasal and oral routes provide 5 to 20% of the delivery efficiency achieved by the i.p. injection, i.e., in the experimental conditions mean concentration of the oligonucleotides in the blood was 3 µ*M* in the former vs. 15 µ*M* in the latter case. These concentrations fit the concentration range required for the oligonucleotide derivatives to affect target viral and cellular nucleic acids. In the cases of intravaginal and rectal administration of oligonucleotides, the concentrations of the labeled compound in organs was 5 to 10 times less than that achieved by intranasal and oral routes. Application of the aqueous oligonucleotide solution onto the skin resulted in much lower efficacy of

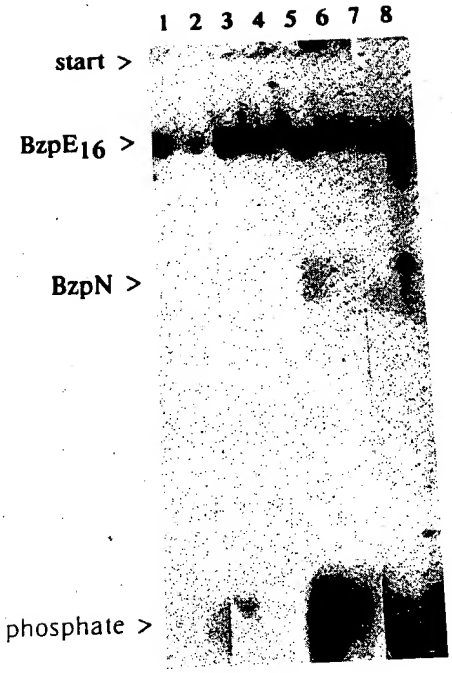

Figure 3 Stability of oligonucleotide derivatives introduced in mice by different routes. The experimental conditions were as described in the legend to Figure 2. The radioactivity of the blood samples (odd-numbered lanes on the gel) and pancreas samples (even-numbered lanes) was analyzed by electrophoresis under denaturing conditions (20% PAAG, 7 *M* urea). 1, 2, Intranasal administration; 3, 4, intavaginal administration; 5, 6, administration per os; 7, 8, i.p. injection. $BzpE_{16}$, position of the initial oligonucleotide derivative; BzpN, position of the mononucleotide derivative. (From Vlassov, V.V., Karamyshev, V.N., and Yakubov, L.A., FEBS Lett., 327, 271, 1993. With permission.)

delivery in the organism.[27] All the administration routes tested provided similar oligonucleotide distribution among organs. It suggests that there is one mechanism of transport of the oligonucleotides into organs: transportation by the bloodstream followed by uptake by cells.

Electrophoretic analysis has revealed that oligonucleotides reach various organs and tissues of the animals less degraded as compared to the i.p.-injected compounds.

Stability of the oligonucleotide derivatives in animal organisms depends considerably on the route of administration used: the oligonucleotide derivative tested remains practically undegraded in the organism for 30 min when being introduced intranasally, intravaginally, and per rectum, while in the case of intraperitoneal degradation of the compound reaches 50% at the same time.

Figure 3 shows results of electrophoretic analysis of the radioactive material isolated from blood and pancreas of mice 20 min after the administration of the labeled oligonucleotide derivatives. It is seen that in all the samples undegraded oligonucleotides are present in reasonable amounts. Accumulation of the labeled inorganic phosphate in the blood was observed when the oligonucleotides were administered intraperitoneally and per os (up to 70% of the total radioactivity 20 min postinjection). Approximately 15% of the recovered radioactivity in the case of intraperitoneal injection and 3% in the case of administration per os was associated with partially degraded oligonucleotides, whereas in the case of the intravaginal and intranasal injection, no products of the oligonucleotide

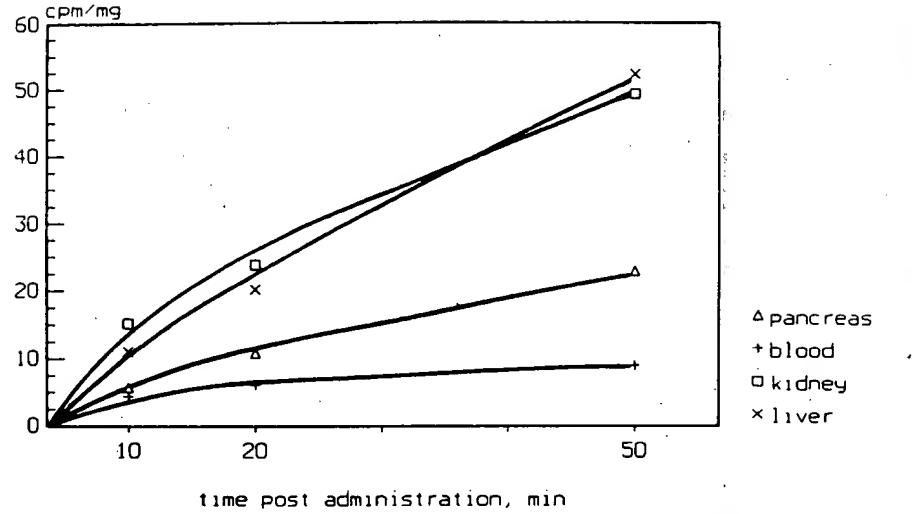

Figure 4 Time course of oligonucleotide accumulation in mice organs after the nasal administration. Experimental conditions were as described in the legend to Figure 2 except for the times.

degradation were detected (only 3% of the inorganic phosphate). This can be explained by the increased activity of macrophages and the presence of nucleases in the peritoneal cavity and intestines, which degrade oligonucleotides before they reach the bloodstream. In the pancreas, degradation of the oligonucleotides seems somewhat slower than in the blood: 30, 45, 70, and 95% of the oligonucleotides remained undegraded in the case of the i.p., peroral, intranasal, and intravaginal injections, respectively. This suggests that the pancreatic cells select preferentially undegraded oligonucleotides from the blood, perhaps by using some specific receptors. It was found that the CD4 receptor binds long oligonucleotides more tightly than the short ones, and that the oligonucleotides shorter than 5 mer bind to the receptor very poorly.[13] In the case of the intravaginal application, a less pronounced degradation of the oligonucleotides was observed. The obtained results have shown that the nasal and ocular routes can provide reasonable efficiency of delivery of oligonucleotides. We investigated administration of oligonucleotides by these and ocular routes in more detail. We also attempted to increase efficiency of the transdermal route by using detergent lotions and iontophoresis.

2. Intranasal Administration

The use of the nasal cavity as an alternative route of delivery has received much attention due to the ease of administration. We have found that oligonucleotides can be delivered by this route quite efficiently. Kinetics of the process (Figure 4) and the nonlinear dependence of the absorbed amount of the compound on the applied dose suggested that it is not a passive diffusion, but rather an active saturable transport through a biological barrier. At high oligonucleotide doses applied (5 nmol per animal) radioactivity continued to accumulate in animals for up to 2 h after the application.

3. Ocular Administration

We have found that administration of oligonucleotides in the form of eye drops was followed by absorption of the compounds and distribution in the organism. Figure 5 shows the kinetics of accumulation of the radiolabeled derivatives of an oligonucleotide in mouse organism after ocular administration. Concentration of the compound in the bloodstream

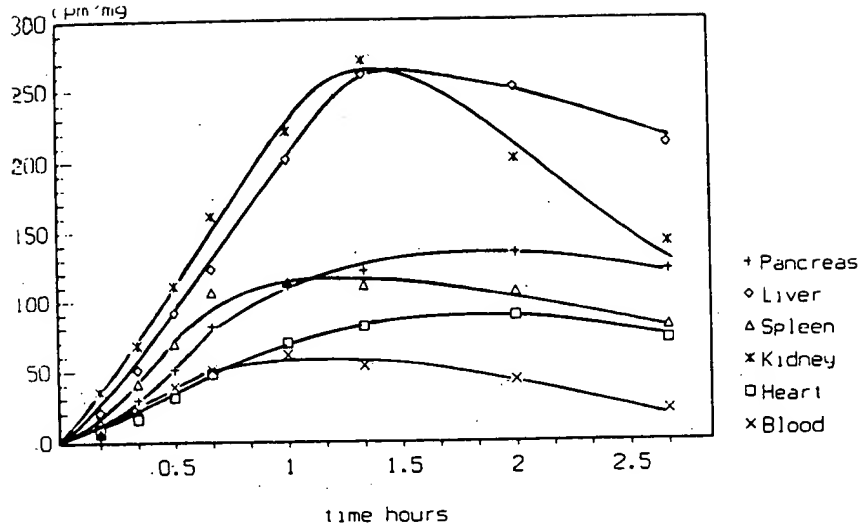

Figure 5 Kinetics of oligonucleotide accumulation in mice organs after the ocular administration. 2 μl 150 μ*M* ^{32}P $BzpE_{16}$ were administered through mouse eye. After certain time points the mice were decapitated, and the radioactivity of blood and sample organs was determined in a liquid scintillation counter. X, blood; □, heart; *, kidney; △, spleen; ◇, liver; +, pancreas.

reaches its maximum within 1 h. The pattern of the oligonucleotide distribution after its absorption seems to be similar to that produced by other methods tested. The applied dose-absorbed amount curve showed a dependence similar to that observed for the intranasal route, also suggesting the existence of a saturable absorption mechanism.

To learn more about the mechanism of transportation of oligonucleotide through the eye we investigated the dependence of the process efficiency on the size of the oligonucleotide. We administered by ocular route derivatives of oligonucleotides of various length: 4, 10, 16, and 22 mers. Figure 6 shows the results of the experiments. Ocular route provides 10% of the penetration efficiency achieved by the intraperitoneal injection for all the oligonucleotides tested. The delay in the appearance of the compounds in the organism is an apparent result of the complicated pathway of the compounds through the mucous membrane in conjunctiva and in the nasolacrimal system: 30 min after administration only a small part of the material applied accumulates in the mice organism (Figure 5). Results of the experiment show that longer oligonucleotides penetrate by the ocular route more easily. This dependence is opposite to the one known for peptides introduced by the ocular route.[28] The fact may suggest the existence of the transcytosis mechanism of the oligonucleotide absorption through the eye, in contrast to the diffusion for peptides. Specific nucleic acid-binding receptors could provide such a mechanism, which can also explain the length dependence of the delivery efficiency because long oligonucleotides bind to the receptors more tightly than short ones.

It was found that simultaneous application of the oligonucleotides and competitors such as sonicated DNA (5 mg/ml), unlabeled 16-mer oligonucleotide (1 m*M*) and heparane sulfate (1 m*M*) inhibited the oligonucleotide uptake. These results are evidence in favor of the active oligonucleotide transportation through the eye.

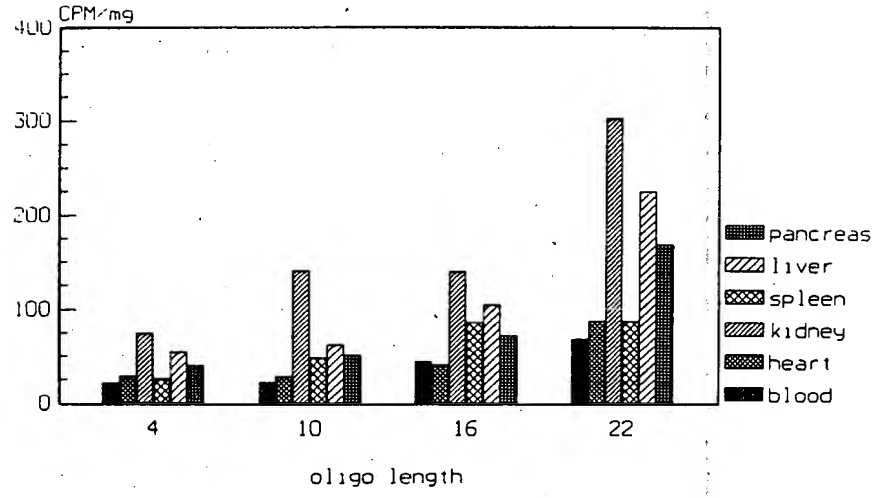

Figure 6 Dependence of delivery efficiency on the oligonucleotide length by the ocular route. 0.3 n*M* (4.4 mln cpm) of each oligonucleotide were administered in mice through the eye. After 30 min the radioactivity of the organ samples was determined.

4. Transdermal Administration

Permeation of oligonucleotides across the mammalian skin could be expected to be very poor. Indeed, when the compounds were applied in water, this route was found to be the least efficient. However, when a mild detergent was included in the solution and the compound was applied in the form of a lotion composed of equal volumes of water, glycerol, and Tween® 80, the penetration efficiency was increased by an order of magnitude and reached the level of the administration routes through mucosa.[27] Further modifications of the solution composition may result in further enhancement of the efficiency of the process. Penetration of ionic oligonucleotides through the unperturbed skin can take place only via hydrophilic pathways. Logically, it could then be influenced by an electric field because of the anionic nature of oligonucleotides, and we attempted to use iontophoresis for facilitating the percutaneous flux of oligonucleotides.

5. Iontophoresis

There are several advantages of iontophoresis over classical transdermal patches: iontophoresis yields much higher efficacy of drug delivery and it provides a means of manipulating the rate of the process. We performed iontophoresis across mouse skin in salt solution. An aluminum cathode was placed under the 1.5- × 1.5-cm Whatman® 3MM paper soaked with 100 µl of the solution containing 0.15 *M* NaCl and 2 nmol (2×10^7 cpm) of radiolabeled oligonucleotide and was applied at the stomach of the immobilized mouse. An aluminum anode with the paper was put on the wet back of the mouse. Electrophoresis was run for 20 min at 2 mA. After the procedure, we observed a considerable amount of oligonucleotides in all organs of the animal. The distribution pattern was similar to that observed by using other routes tested. The efficiency of administration far exceeded (by a factor of 20 under the conditions used) that achieved by simple application of the oligonucleotide solution at the animal skin. It was inferred from this study that controlled transdermal administration is possible through this approach.[27]

We tried to deliver oligonucleotides by means of iontophoresis into a solid tumor of a living mouse. The experiment has been performed with C3H mice with spontaneous subdermal mammary gland tumors. The electrophoretic procedure was performed as

described above, except that the cathode was applied on the region of the tumor and electrophoresis was run for 50 min. After the procedure, distribution of the compounds in the animal body and in different parts of the tumor was determined. It was found that the oligonucleotide entered the tumor and further distributed among the animal organs as well as in the healthy animals. The surface layers of the tumor which were close to the electrode accumulated more oligonucleotides as compared to its opposite part. The mean concentration of the oligonucleotide in the tumor was as high as in the liver, which retains the compounds actively. This result evidences that increased local concentration of oligonucleotides can be created in solid tumor tissue by means of iontophoresis directed in the tumor.

IV. CONCLUSIONS

The described experiments evidence that oligonucleotides can be delivired into mice by the intranasal, ocular, rectal, and vaginal routes and even through the skin. Taking into account the existence of specific nucleic acid-binding cellular receptors, we suggest that the mechanism of oligonucleotide penetration is transcytosis through the mucosa cells. Our studies have shown that it is possible by using alternative strategies to obtain clinically relevant levels of administration via the nasal and ocular routes without the necessity to change the structure of the oligonucleotides or destroying the epithelial membrane. We have found that the transdermal delivery of oligonucleotides can be improved considerably by using iontophoresis. Oligonucleotides delivered by the above mentioned administration routes reach animal organs in a biologically intact form. This makes one optimistic about the prospects of the *in vivo* use of natural oligonucleotides.

ARIES

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EXHIBIT 5

DRUG DISPOSITION

Clin. Pharmacokinet. 28 (1): 7-16, 1995
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Pharmacokinetics of Antisense Oligonucleotides

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Contents

Summary

Antisense oligonucleotides are promising therapeutic agents for the treatment of life-threatening diseases.

Intravenous injection of phosphodiester oligonucleotide analogue (P-oligonucleotide) in monkeys shows that the oligonucleotide is degraded rapidly in the plasma with a half-life of about 5 minutes. Administration of a single dose of the phosphorothioate (S-oligonucleotide) in animals by the intravenous route reveals biphasic plasma elimination. An initial short half-life (0.53 to 0.83 hours) represents distribution out of the plasma compartment and a second long half-life (35 to 50 hours) represents elimination from the body. This elimination half-life was similar when the oligonucleotide was administered subcutaneously. In contrast, methylphosphonate oligonucleotides have an elimination half-life of 17 minutes in mice.

S-Oligonucleotide was distributed into most of organs of rats and mice. Liver and kidney were the 2 organs with highest uptake of the oligonucleotide. The S-oligonucleotide was primarily excreted in urine. Up to 30% was excreted in the first 24 hours.

Repeated daily intravenous injections of a 25-mer S-oligonucleotide into rats showed that the concentrations in the plasma are at steady-state during the 8 days' administration.

The data represented here support the potential utility of phosphorothioate and methylphosphonate oligonucleotides as therapeutic agents *in vivo*.

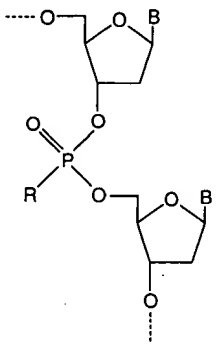
The antisense oligonucleotide–based therapeutic approach holds promise as treatment modalities for life-threatening disease. Antisense oligonucleotides have attracted special interest as a novel class of therapeutic agents for the treatment of cancer, viral infections and genetic disorders, because of their ability to inhibit gene expression in a sequence-specific manner.[1] Antisense oligonucleotides are designed to hybridise to the disease-associated mRNA, thereby inhibiting translation of mRNA into proteins. The potential of antisense oligonucleotides to regulate gene expression has been shown *in vitro* and *in vivo* against various gene targets.[2-6]

Three oligonucleotide analogues that have been studied in detail are oligonucleotides containing phosphodiester, phosphorothioate and methylphosphonate internucleotide linkages (fig. 1). Phosphorothioate analogues have a sulphur substitution for one of the non-bridged oxygens at the internucleotide linkage and are therefore known as S-oligonucleotides.[7] Phosphorothioates have been found to be effective inhibitors of HIV, influenza virus, herpes simplex virus, cytomegalovirus, Epstein Barr virus, hepatitis B virus and human papilloma virus.[8] Methylphosphonate analogues are non-ionic and contain a methyl group substitution for one of the non-bridged oxygen–oligonucleotide linkages.[9] Methylphosphonate analogues have been found to be inhibitors of herpes simplex virus[9] and HIV.[10]

There have been a number of reports describing the chemistry and biochemical properties of oligonucleotides containing phosphodiester, phosphorothioate and methylphosphonate analogues.[2-6] This article primarily describes the pharmacokinetics and *in vivo* stability of S-oligonucleotide in mice, rats, and monkeys.

1. Synthesis of Oligonucleotides

Oligonucleotides containing phosphodiester (P-oligonucleotides) as well as phosphorothioate (S-oligonucleotides) linkages were synthesised using either β-cyanoethyl phosphoramidite chemistry or H-phosphonate chemistry on an automated synthesiser.[11] ^{35}S-Labelled S-oligonucleotide was synthesised by using H-phosphonate chemistry, by the same procedure reported by other investigators.[12] Two sequences are discussed in this review: a 20-mer (5′-ACACCCAATTCTGAAAATGG) complementary to the *tat* splice acceptor site of HIV-1[12] and a 25-mer (5′-CTCTCGCACCCATCTCTCTCCTTCT), complementary to the AUG site of the *gag* region of HIV-1. Both oligonucleotides have been found to inhibit HIV-1 replication *in vitro*,[13,14] and the 25-mer (named Gene Expression Modulator 91; GEM91, a S-oligonucleotide) is presently undergoing clinical trials.



R = –O; Phosphodiester oligonucleotide
–S; Phosphorothioate oligonucleotide
CH_3; Methylphosphonate oligonucleotide

Fig. 1. Structural formulae of oligonucleotide analogues.

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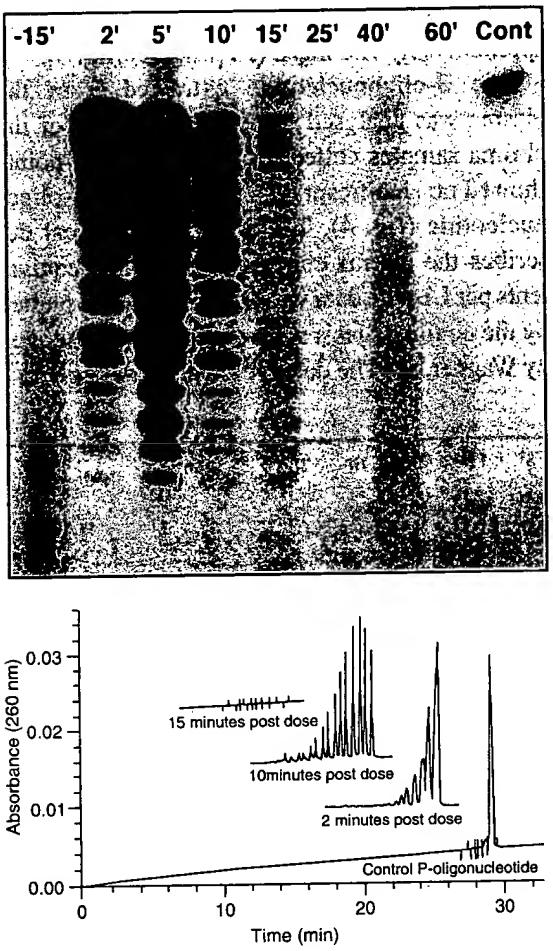

Fig. 2. *In vivo* stability of phosphodiester oligonucleotides (P-oligonucleotides) 2, 5, 10, 15, 25, 40 and 60 minutes after intravenous administration of 30 mg/kg to a monkey. Oligonucleotide was extracted and analysed by: **(top)** polyacrylamide gel electrophoresis (PAGE); and **(bottom)** capillary gel electrophoresis (CGE). For analysis by PAGE, the extracted P-oligonucleotide was ^{32}P-labelled at the 5'-end. For CGE, P-oligonucleotide was extracted and analysed.

2. Pharmacokinetics of Phosphodiester Oligonucleotides in Monkeys

25-Mer P-oligonucleotide was injected intravenously in monkeys at a dose of 30 mg/kg. Blood samples were withdrawn at 2, 5, 10, 15, 25, 40 and 60 minutes postdose in heparinised tubes and stored at –20°C. 25-Mer P-oligonucleotide present in the blood samples was extracted by a phenol-chloroform extraction procedure.[12] The extracted P-oligonucleotide was analysed by polyacrylamide gel electrophoresis (PAGE), and also by capillary gel electrophoresis (CGE). For PAGE analysis, extracted P-oligonucleotides were labelled with ^{32}P by using γ-^{32}P adenosine triphosphate (ATP) and T4 polynucleotide kinase. After electrophoresis the gel was autoradiographed.

Analysis by PAGE showed that within 5 minutes of intravenous administration about 50% of the administered P-oligonucleotide had been degraded, and after 15 minutes all of the P-oligonucleotide was degraded (fig. 2). Analysis by CGE also demonstrated similar results (fig. 2). These results indicate that the half-life of degradation of P-oligonucleotide *in vivo* (in monkey plasma) is about 5 minutes. Similar results have been reported for a 20-mer (^{3}H-labelled) P-oligonucleotide in rats.[15]

3. Pharmacokinetics of Methylphosphonate Oligonucleotides in Mice

There has been a short report on the disposition of oligonucleotide methylphosphonate.[16]

Following a single intravenous injection of a ^{3}H-labelled 12-mer oligonucleotide methylphosphonate into mice, the plasma half-life corresponding to the distribution and elimination phases were 6 and 17 minutes, respectively.

Radioactivity was excreted primarily in urine, about 70% of the total administered radioactivity was present in urine between 60 and 120 minutes after administration of the methylphosphonate oligonucleotide. Peak concentrations of radioactivity were observed in most tissues 5 minutes after administration of the oligonucleotide. Highest concentrations were found in renal tissue, while lung, liver, spleen, muscle and brain had a lower tissue concentration. Oligonucleotide was metabolised very slowly; indeed, the intact 12-mer molecule was identified in excreted samples of urine.

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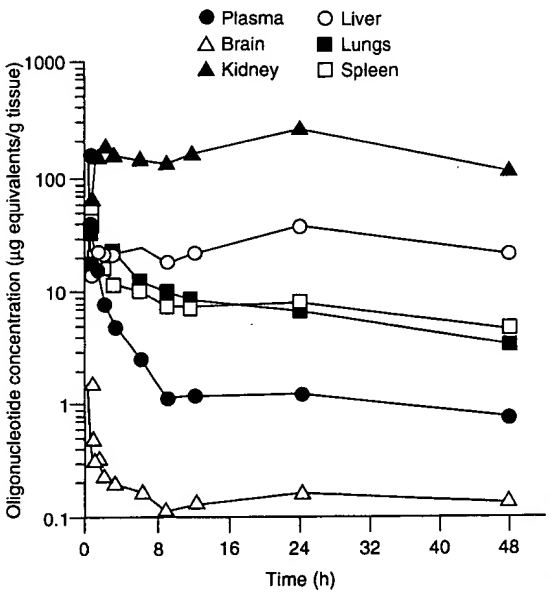

Fig. 3. Plasma clearance and tissue distribution of 20-mer phosphorothioate oligonucleotide (S-oligonucleotide) after intravenous administration of 30 mg/kg in mice. The concentration of oligonucleotide represented here is in µg equivalents, on the basis of radioactivity.

4. Pharmacokinetics of S-Oligonucleotides in Mice

A single dose of 30 mg/kg of 20-mer S-oligonucleotide (specific activity 8.02 µCi/mg in 0.2ml volume) was administered intravenously via the tail vein of male CD2F1 mice (weight 20g). The test animals were sacrificed and tissues were removed for quantitation of the ^{35}S-radioactivity 5, 15, and 30 minutes, and 1, 2, 3, 6, 9, 12, 24 and 48 hours after administration of the drug. The organs were homogenised in deionised water, and diluted 1 : 10 prior to determination of radioactivity. Urine samples were collected from 0 to 6 hours, 6 to 12 hours and 12 to 24 hours following administration.

4.1 Plasma Pharmacokinetics

Plasma concentrations of S-oligonucleotides were rapidly depleted after intravenous administration; there was concomitant accumulation of S-oligonucleotide in the tissues. After a single dose of 30 mg/kg, the maximum plasma concentration was approximately 130 mg/L (20 µmol/L) [fig. 3].

The S-oligonucleotide extracted from the plasma was analysed by PAGE. Analysis of the plasma samples collected at various time points showed no significant degradation of the S-oligonucleotide (fig. 4). The equation that best describes the plasma concentration (in mg equivalents per L)-time data were determined and plotted by the nonlinear least square method, as described by Wagner.[17] The data were best described by a

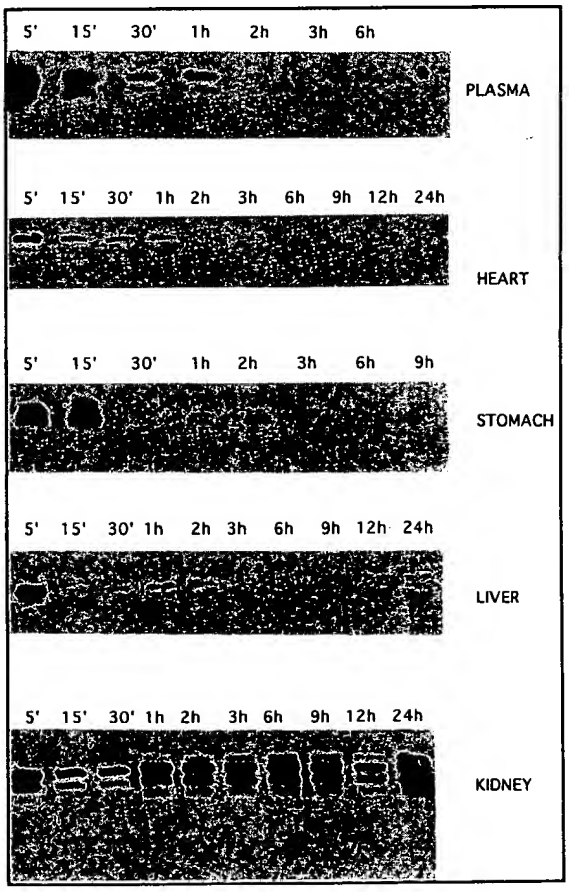

Fig. 4. Polyacrylamide gel electrophoresis of extracted ^{35}S-phosphorothioate oligonucleotides (S-oligonucleotides) from plasma, heart, stomach, liver and kidney after intravenous administration (30 mg/kg) in mice. ^{35}S-Labelled oligonucleotide was extracted and, after electrophoresis, the gel was autoradiographed.

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2-compartment model in which the α-phase elimination half-life was 0.53 hour and the β-phase elimination half-life was 40 hours.

The distribution, elimination and metabolism of 20-mer S-oligonucleotide were also studied after intraperitoneal administration. A single dose of 30 mg/kg resulted in a maximum plasma concentration of 4 μmol/L 1 hour after administration. The plasma elimination half-life was found to be the same as that following intravenous administration.

4.2 Tissue Distribution

After intravenous administration, 20-mer S-oligonucleotide was taken up by most tissues.[12]

The concentration of ^{35}S-oligonucleotide in liver and kidney was markedly higher than in other tissues. The S-oligonucleotide concentration increased in these 2 organs for up to 48 hours (the end-point of the study).

In the other tissues studied, there was elimination of the S-oligonucleotide from the time of initial peak tissue concentrations that paralleled plasma clearance. The concentration of 20-mer S-oligonucleotide in the brain was significantly lower than in other tissues.

Gel electrophoresis of 20-mer ^{35}S-oligonucleotide recovered from the tissues displayed a tissue-specific degradation profile. S-Oligonucleotide was found to be very stable in most tissues except the kidney and liver. The degradation rate in kidney and liver tissues was time-dependent and 50% of the S-oligonucleotide was found to be degraded in 48 hours. In addition, analysis of extracted oligonucleotide from kidney, liver and intestine by gel electrophoresis showed bands with slower mobility than that of the administered oligonucleotide, which suggests either an increase in molecular weight or a reduction in charge of the administered oligonucleotide.

About 30% of the administered dose of S-oligonucleotide was excreted in urine in first 24 hours after administration. Less than 1% of the S-oligonucleotide was excreted in faeces during the first 24 hours. Most of the excreted S-oligonucleotide was found to be degraded.

5. Pharmacokinetics of S-Oligonucleotides in Rats

The distribution, metabolism and excretion of S-oligonucleotide was studied in Sprague-Dawley rats (200g) following intravenous and subcutaneous administration. 25-Mer ^{35}S-oligonucleotide was injected into 3 rats at a dose of 30 mg/kg (10 μCi per animal). Plasma samples were collected at 5 and 15 minutes, and 1, 2, 3, 6, 12, 24, 48, 72 and 96 hours, and radioactivity was determined.

5.1 Single-Dose Plasma Pharmacokinetics in Rats

The initial plasma elimination half-life ranged from 0.83 to 1.11 hours following intravenous administration, and was 2.4 hours following subcutaneous administration (fig. 5). The terminal elimination half-life following intravenous administration ranged from 33.45 to 35.15 hours. Following subcutaneous administration, the terminal elimination half-life was similar to that reported after intravenous administration.

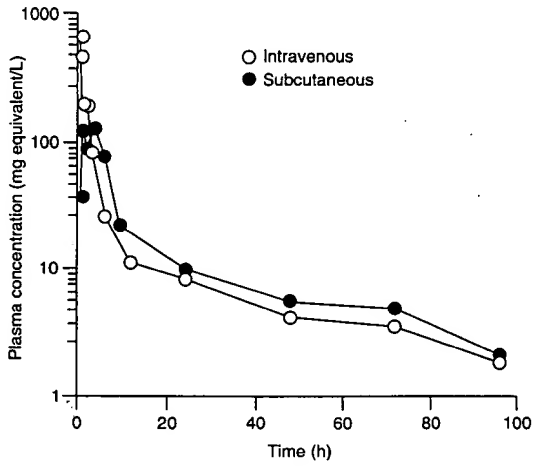

Fig. 5. Comparative plasma concentrations after intravenous and subcutaneous administration of phoshorothioate oligonucleotide (S-oligonucleotide) in rats. The concentration of oligonucleotides is in mg equivalents, based on ^{35}S-radioactivity.

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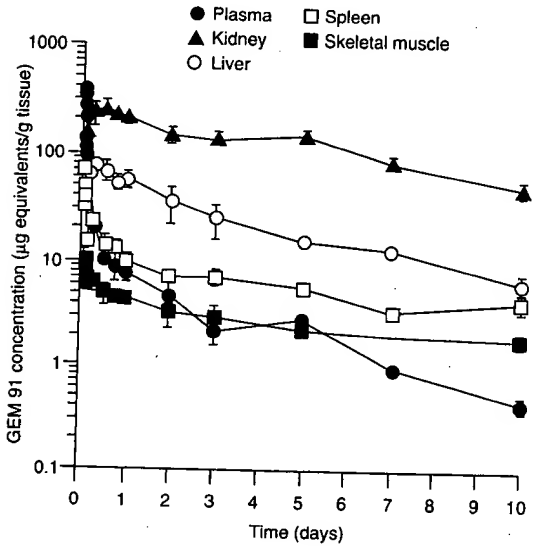

Fig. 6. Plasma clearance and tissue distribution of 25-mer phosphorothioate oligonucleotide [S-oligonucleotide; Gene Expression Modulator 91 (GEM 91)] after intravenous administration of 30 mg/kg in rats. The concentration of oligonucleotide represented here is in µg equivalents on the basis of ^{35}S-radioactivity.

Total body clearance was 24.5 ± 1.9 ml/kg/h for the intravenous dose and 25.3 ± 0.9 ml/kg/h for the subcutaneous dose.

5.2 Tissue Distribution in Rats

Tissue distribution of S-oligonucleotide in rats was similar to that reported in the mouse study. Liver and kidney were the 2 organs with the highest uptake of the ^{35}S-oligonucleotide. The activity associated with liver and kidney followed a similar profile as in plasma, suggesting rapid distribution of S-oligonucleotides. After subcutaneous administration, tissue distribution of the S-oligonucleotide was very similar to that obtained after intravenous administration (fig. 6).

S-Oligonucleotide in rats was primarily excreted in urine; up to 26% of the administered dose was excreted within 24 hours and 58% was excreted over a period of 240 hours. Approximately 10% of the dose was excreted in faeces.

5.3 Multiple-Dose Pharmacokinetics

We have also determined the plasma concentration of 25-mer S-oligonucleotide after daily intravenous administration to rats for 8 days. Doses of 1 mg/kg/day (1 µCi) were given intravenously through the tail vein in 5 male Sprague-Dawley rats (weight range 311 to 392g). During the 8-day administration period, samples of blood (approximately 0.1ml) were taken via the orbital sinus, immediately prior to the next administration of S-oligonucleotide. Slightly larger aliquots (0.5ml) of blood were taken on days 9, 10 and 11. Saline was added to the red blood cells, which were then haemolysed by freezing and thawing.

Figure 7 shows the plasma concentration of S-oligonucleotide 24 hours after administration of the dose, just prior to administration of the next dose. Plasma concentrations of S-oligonucleotide remained relatively flat [0.2 mg equivalents/L (0.02 µmol/L)] during the 8-day administration period. A decrease in the plasma concentration was observed on the second and third days (days 10 and

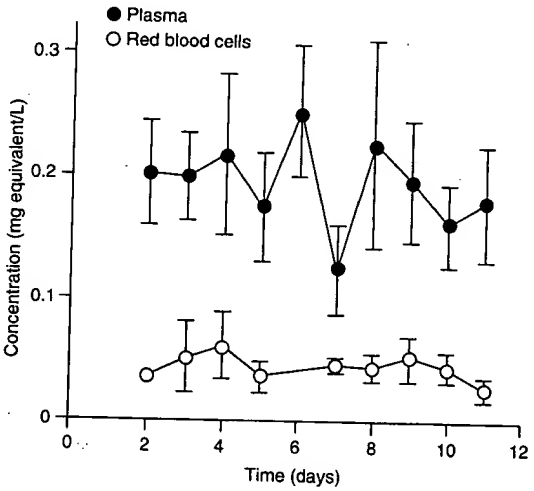

Fig. 7. Concentration of 25-mer phosphorothioate oligonucleotide (S-oligonucleotide) in plasma and red blood cells after daily intravenous administration (1 mg/kg) in rats. The concentrations are in mg equivalents, on the basis of radioactivity, and are taken 24 hours postdose. The bars represent the standard deviation of the mean values from 5 animals.

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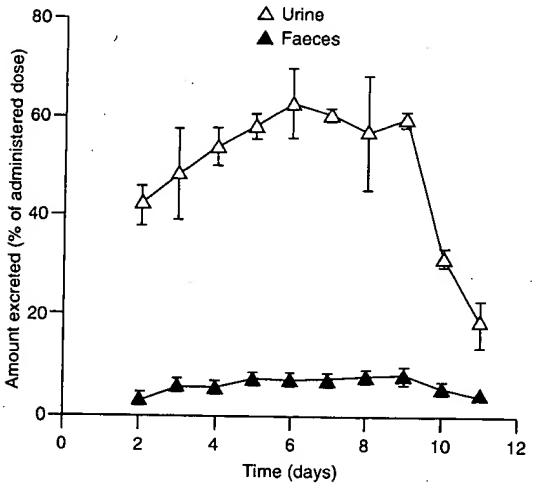

Fig. 8. Urinary and faecal clearance of phosphorothioate oligonucleotide (S-oligonucleotide) after intravenous administration of 25-mer S-oligonucleotide in rats. The bars represent the standard deviation of the mean values from 5 animals.

11), and after the last dose (day 8). The concentration of radioactivity in red blood cell haemolysate was approximately 5-fold lower than that in plasma.

Urine and faeces were also collected from each animal for 11 days. The excretion pattern of ^{35}S-oligonucleotide is shown in figure 8. The major route of excretion was via the urine. The percentage of the dose excreted in urine increased from 42% on day 2 to 62% on day 4 and remained around 60% during days 4 through 9. The percentage decreased on day 10 and 11. A similar trend of excretion, but at much lower levels was seen in the faeces.

6. Pharmacokinetics of S-Oligonucleotides in Monkeys

We have administered a single dose of 1 and 5 mg/kg of 25-mer S-oligonucleotide intravenously in a peripheral vein of Rhesus monkeys. The maximal plasma concentration attained with a single dose of 1 and 5 mg/kg was approximately 0.5 and 2 μmol/L, respectively (fig. 9). The initial and terminal phase elimination half-life were 0.6 to 1 hour and 42.2 to 56.3 hours, respectively, on the basis of the ^{35}S equivalent level. The area under the plasma concentration-time curve also appears to be linear and proportional to the dose administered. The area under the concentration-time curve for a 1 mg/kg dose was 104.2 and 108.4 mg/L · h and for a dose of 5 mg/kg was between 406.1 and 488.0 mg/L · h.

The percentage of the dose of S-oligonucleotide excreted in urine over 96 hours were 27.7 and 53.0% for doses of 1 and 5 mg/kg, respectively.

7. Pharmacokinetics of S-Oligonucleotides in Humans

We have administered a single dose of S-oligonucleotide (^{35}S-labelled; 25-mer) into 6 HIV-1 infected individuals by 2-hour intravenous infusion at a dose of 0.1 mg/kg and assessed the plasma clearance profile and urinary excretion.[18] Plasma disappearance curve for 25-mer S-oligonucleotide could be described by the sum of 2 exponentials with mean half-lives of 0.18 (± 0.04) and 26.71 (± 1.67) hours based on radioactivity levels. Urinary excretion represented the major pathway of

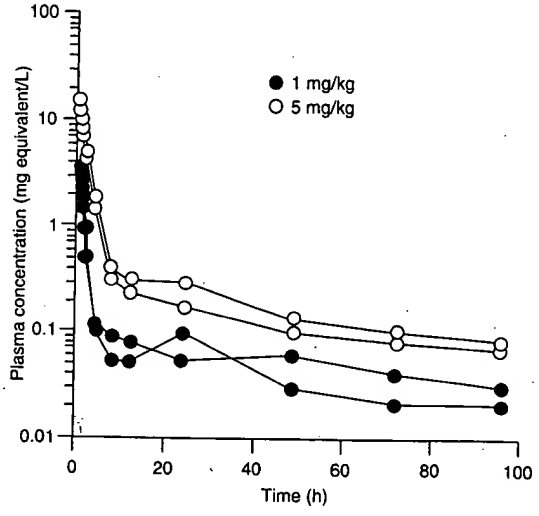

Fig. 9. Plasma clearance of phosphorothioate oligonucleotide (S-oligonucleotide) after intravenous administration of both 1 and 5 mg/kg in 2 monkeys.

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elimination of S-oligonucleotide. On the basis of radioactivity levels, 49.15 ± 6.80% of the administered dose was excreted within 24 hours and 70.37 ± 6.72% within 96 hours of S-oligonucleotide administration.

Analysis of the extracted radioactivity in plasma showed both intact and degraded form of S-oligonucleotide; however, in urine all the radioactivity was associated with the degraded form of the S-oligonucleotide. In an independent study, 20-mer S-oligonucleotide was administered at a dose of 0.05 mg/kg/hour for 10 days in 5 individuals with either relapsed or refractory acute myelogenous leukaemia (AML) or myelodysplastic syndrome (MSD).[19] 30% to 62% of the administered dose of the oligonucleotide was excreted in urine within 10 days. The plasma concentration ranged from 2.1 mg/L (0.32 μmol/L) to 6.4 mg/L (0.97 μmol/L).

8. Implications for Clinical Application of Oligonucleotides and Conclusion

The half-life of degradation of oligonucleotides-containing phosphodiester internucleotide linkages is about 5 minutes, which limits their use as drugs.[15,20] However, phosphorothioate analogues of oligonucleotide have long half-lives[12,15,21-23] of degradation and, therefore, long plasma half-lives. Once S-oligonucleotide enters the systemic circulation a rapid distribution into tissues is observed. The S-oligonucleotide was distributed in most major organs, with brain having the lowest concentration.

Degradation of S-oligonucleotide was found to be tissue-dependent. The degradation of oligonucleotide appears to be primarily from the 3′-end, as 3′-end modified oligonucleotide phosphorothioates resist degradation *in vivo*.[24] This also appears to be the case for P-oligonucleotides.[25] The pattern of tissue uptake, distribution and elimination was similar for phosphorothioates of varying length and base composition.[12,15,21,22] Pharmacokinetic study of a 12-mer methylphosphonate oligonucleotide showed that the elimination half-life in plasma was 6 minutes, and about 70% of the administered oligonucleotide was excreted in urine within 2 hours postdose.[16] Three different routes of administration have been studied: (i) intravenous; (ii) subcutaneous; and (iii) intraperitoneal. The S-oligonucleotide is found to be bioavailable after administration by all 3 routes.

Various procedures have been established to study the pharmacokinetics of S-oligonucleotide. In most studies, radiolabelled oligonucleotide has been used, and oligonucleotide tissue concentrations have been reported as radioactivity equivalents. The concentration of degree of degradation of oligonucleotides can be quantitated in biological fluids by various methods other than radiolabelling. The methods include the following: (i) high performance liquid chromatography (HPLC) analysis;[21,16] (ii) labelling of extracted oligonucleotides with ^{32}P followed by gel electrophoresis or HPLC; (iii) analysis by capillary gel electrophoresis; or (iv) crosslinking of extracted oligonucleotide onto solid phase followed by hybridisation with oligonucleotide probe.[26] In all cases the sensitivity of detection is in pmol/L range.

Although the antisense therapeutic approach was first proposed by Zamecnik and Stephenson in 1978,[27] only during the past 4 years have *in vivo* studies have been reported for oligonucleotides. The results of *in vitro* studies, in which the drug concentrations and metabolism may or may not mimic *in vivo* conditions, demonstrate that antisense oligonucleotides have significant antiviral activity.[8] In order for antisense oligonucleotides to have potential as effective therapeutic agents, appropriate drug concentrations must be attained and maintained *in vivo* at the site of drug action. Nuclease-resistant backbones, such as the phosphorothioate or methylphosphonate molecules, confer resistance to degradation so that a biologically active drug can reach the intended site of action as a biologically active substance. However, other factors such as the distribution, metabolism and excretion, and the rates at which these events occur, also determine the therapeutic utility of a drug.

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The findings reviewed in this paper and the results of similar studies[12,15,21,22] demonstrate that S-oligonucleotides are rapidly taken up into the tissues following intravenous drug administration. This is followed by an elimination phase with a long elimination half-life. The S-oligonucleotides are primarily excreted in the urine. The distribution pattern is similar in the mouse, rat and monkey. Not much is known about the *in vivo* metabolic fate of oligonucleotides or their analogues. Most of the oligonucleotide excreted in urine is degraded. It has been found that oligonucleotides are metabolised so that up to 50% of the radioactivity (of ^{14}C-labelled S-oligonucleotide) is exhaled in air.[21] We have reported earlier that higher molecular weight bands were observed when extracted S-oligonucleotide was analysed by gel electrophoresis; however, the identity of the molecules in this high molecular weight band has not yet been confirmed.[12]

The role of base composition on metabolism and pharmacokinetics also requires additional study. PAGE and radiochromatograms show that oligonucleotides with nuclease-resistant backbones can be degraded *in vivo*, and that the liver and kidney are the principle organs of metabolism. The pattern of oligonucleotide distribution and excretion has been studied in various species. However, more remains to be learned about the identity of the metabolites, the rates at which they are formed, and the enzymes responsible for oligonucleotide metabolism.

The data presented here suggest that P-oligonucleotides would have limited therapeutic utility due to their rapid degradation *in vivo*. The S-oligonucleotides, however, have favourable absorption and distribution kinetics and sufficient *in vivo* stability to be used as therapeutic agents. The relatively long elimination half-life in plasma suggests that infrequent administration could be used to maintain a therapeutically effective concentration of S-oligonucleotides.

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EXHIBIT 6

Crooke, Therapeutic Applications of Oligonucleotides, R.G. Landers Co., Austin, TX, 1995, Chapter 5, Pages 63-84

CHAPTER 5

PHOSPHOROTHIOATE OLIGONUCLEOTIDES

INTRODUCTION

Of the first generation oligonucleotide analogs, the class that has resulted in the broadest range of activities and about which the most is known, is the phosphorothioate class. Phosphorothioate oligonucleotides were first synthesized in 1969 when a poly rI:poly rC phosphorothioate was synthesized (De Clerq et al, 1969). This modification clearly achieves the objective of increased nuclease stability. In this class of oligonucleotides, one of the oxygen atoms in the phosphate group is replaced with a sulfur. The resulting compound is negatively charged, as is a phosphodiester, but is much more resistant to nucleases (Cohen, 1993).

SYNTHESIS AND PURIFICATION

A significant advantage of phosphorothioates is that they can be synthesized on automated synthesizers with relatively minor modifications of the synthetic process. Instead of the usual oxidation step, sulfurization is effected. Elemental sulfur or more useful reagents, such as the Beaucage reagent, can be used (Iyer et al, 1990; Cohen, 1993). During the past several years, remarkable progress on scaling up both automated and solution phase synthesis and reducing the cost of synthesis of phosphorothioates has been achieved to the extent that large preclinical research and development efforts and substantial clinical programs can now be supported. Both large scale automated and solution phase synthetic processes are available (Zon and Geiser, 1991).

Inasmuch as each phosphorothioate group is prochiral, synthetic processes that are not stereospecific result in 2^n isomers where n is the number of phosphorothioate internucleotide linkages. There are modest differences in hybridization of the Rp and Sp isomers with all Sp isomer oligonucleotides having higher affinity for RNA or DNA than the racemic mixture and different nuclease resistance (Eckstein & Gish, 1989; Guga et al, 1987). Significant efforts to develop chiral synthetic efforts have been mounted (Guga et al, 1987; Battisini et al, 1991;

Stec, 1993). To date, however, all significant studies on phosphorothioate oligonucleotides have employed chiral mixtures and, given the large number of isomers and relatively small differences in biological properties of the Rp and Sp isomers, regulatory agencies have accepted applications for clinical trials on the chiral mixtures.

Purification of phosphorothioate oligonucleotides can be achieved using systems similar to those used for phosphodiesters. However, significant modifications in protocol are required to achieve good purification using ion exchange high performance chromatography or capillary electrophoresis (Zon & Geiser, 1991). These compounds can also be purified using reverse phase HPLC prior to deprotection.

The molecular weight of phosphorothioates can be determined by mass spectrometry and protocols have now been developed that allow sequencing with nucleases (Tang et al, 1993; Cummins, unpublished data). Base composition can be determined after digestion with nucleases.

HYBRIDIZATION

The hybridization of phosphorothioate oligonucleotides to DNA and RNA has been thoroughly characterized (Crooke, 1992; Crooke, 1993; see Crooke & Lebleu, 1993 for reviews). The T_m of a phosphorothioate oligodeoxynucleotide for RNA is approximately 0.5°C less per nucleotide than for a corresponding phosphodiester oligodeoxynucleotide. This reduction in T_m per nucleotide is virtually independent of the number of phosphorothioate units substituted for phosphodiesters. However, sequence context has some influence as the ΔT_m can vary from -0.3°C-1.0°C depending on sequence. Compared to RNA:RNA duplex formation, a phosphorothioate oligodeoxynucleotide has a T_m approximately -2.2°C lower per unit (Freier, 1993). This means that to be effective in vitro, phosphorothioate oligodeoxynucleotides must typically be 17-20-mer in length (Monia et al, 1992; Monia et al, 1993) and that invasion of double-stranded regions in RNA is difficult (Vickers et al, 1991; Lima et al, 1992).

Association rates of phosphorothioate oligodeoxynucleotide to unstructured RNA targets are typically 10^6-$10^7 M^{-1}S^{-1}$ independent of oligonucleotide length or sequence (Lima et al, 1992; Freier, 1993). Association rates to structured RNA targets can vary from 10^2-$10^8 M^{-1}S^{-1}$ depending on the structure of the RNA, site of binding in the structure and other factors (Freier, 1993). Said another way, association rates for oligonucleotides that display acceptable affinity constants are sufficient to support biological activity at therapeutically achievable concentrations.

The specificity of hybridization of phosphorothioate oligonucleotides is, in general, slightly greater than phosphodiester analogs. For example, a T-C mismatch results in a 7.7° or 12.8°C reduction in T_m, respectively, for a phosphodiester or phosphorothioate oligodeoxynucleotide 18 nucleotides in length with the mismatch centered (Freier, 1993). Thus, from this perspective, the phosphorothioate modification is quite attractive.

INTERACTIONS WITH PROTEINS

Phosphorothioate oligonucleotides bind to proteins. The interactions with proteins can be divided into non-specific, sequence-specific and structure-specific binding events, each of which may have different characteristics and effects. Non-specific binding to a wide variety of proteins has been demonstrated. Exemplary of this type of binding is the interaction of phosphorothioate oligonucleotides with serum albumin. The affinity of such interactions is low. The Kd for albumin is approximately 400 μM, thus, in a similar range with aspirin or penicillin (Joos & Hall, 1969). Phosphorothioate oligonucleotides can interact with nucleic acid binding protein such as transcription factors and single-strand nucleic acid binding proteins. However, very little is known about these binding events. Additionally, it has been reported that phosphorothioates bind to an 80Kd membrane protein that was suggested to be involved in cellular uptake processes (Loke et al, 1989). However, again, little is known about the affinities, sequence or structure specificities of these putative interactions.

Phosphorothioates interact with nucleases and DNA polymerases. These compounds are slowly metabolized by both endo and exonucleases (Crooke, 1992) and inhibit these enzymes (Graham et al, 1994). The inhibition of these enzymes appears to be competitive and this may account for some early data suggesting that phosphorothioates are almost infinitely stable to nucleases. In these studies, the oligonucleotide to enzyme ratio was very high and, thus, the enzyme was inhibited. Figure 5.1 shows the effects of snake venom phosphodiesterase, a 3' exonuclease, on a phosphorothioate oligonucleotide at three different oligonucleotide concentrations. The cleavage curves are bifunctional, suggesting inhibition of the enzyme by high drug concentrations. Further, a phosphodiester oligonucleotide added after degradation of the phosphorothioate plateaued was not degraded. Clearly, such effects could have significant pharmacokinetic consequences.

Phosphorothioates also bind to RNase H when in an RNA-DNA duplex and the duplex serves as a substrate for RNase H (Gao et al, 1991). At higher concentrations, presumably by binding as a single strand to RNase H, phosphorothioates inhibit the enzyme (Gao et al, 1991). Again, the oligonucleotides appear to be competitive antagonists for the DNA-RNA substrate.

Phosphorothioates have been shown to be competitive inhibitors of DNA polymerase α and β with respect to the DNA template, and non-competitive inhibitors of DNA polymerases γ and δ (Gao et al, 1991). Despite this inhibition, several studies have suggested that phosphorothioates might serve as primers for polymerases and be extended (Stein & Cheng, 1993; Agrawal et al 1991; Graham et al, 1994). In our laboratories, we have shown extensions 2-3 nucleotides only. At present, a full explanation as to why no longer extensions are observed is not available.

Phosphorothioate oligonucleotides have been reported to be competitive inhibitors for HIV-reverse transcriptase (Majumdar et al, 1989) and inhibit reverse transcriptase-associated RNase H activity (Cheng

et al, 1991). They have been reported to bind to the cell surface protein, CD4 (Stein et al, 1991), and to protein kinase C (Fields et al, 1988). Various viral polymerases have also been shown to be inhibited by phosphorothioates (for review, see Stein & Cheng, 1993). Additionally, we have shown potent, non-sequence specific inhibition of RNA splicing by phosphorothioates (Hodges & Crooke, unpublished data).

Like other oligonucleotides, phosphorothioates can adopt a variety of structures. As a general rule, self-complementary oligonucleotides are avoided, if possible, to avoid duplex formation between oligonucleotides. However, other structures that are less well understood can also form. For example, oligonucleotides containing runs of guanosines can form tetrameric structures called G-quartets, and these appear to interact with a number of proteins with relatively greater affinity than unstructured oligonucleotides (Wyatt et al, 1994). ISIS 5320 is exemplary (Fig. 5.2). It is a G-quartet formed of four strands of phosphorothioate octamers of the sequence $T_2G_4T_2$ and binds to the V3 loop of the HIV protein, gp120, with relatively high affinity and prevents viral adsorption and cell-to-cell transmission of the virus (Wyatt et al, 1994).

In conclusion, phosphorothioate oligonucleotides may interact with a wide range of proteins via several types of mechanisms. These interactions may influence the pharmacokinetic, pharmacologic and toxicologic properties of these molecules. They may also complicate studies on the mechanism of action of these drugs.

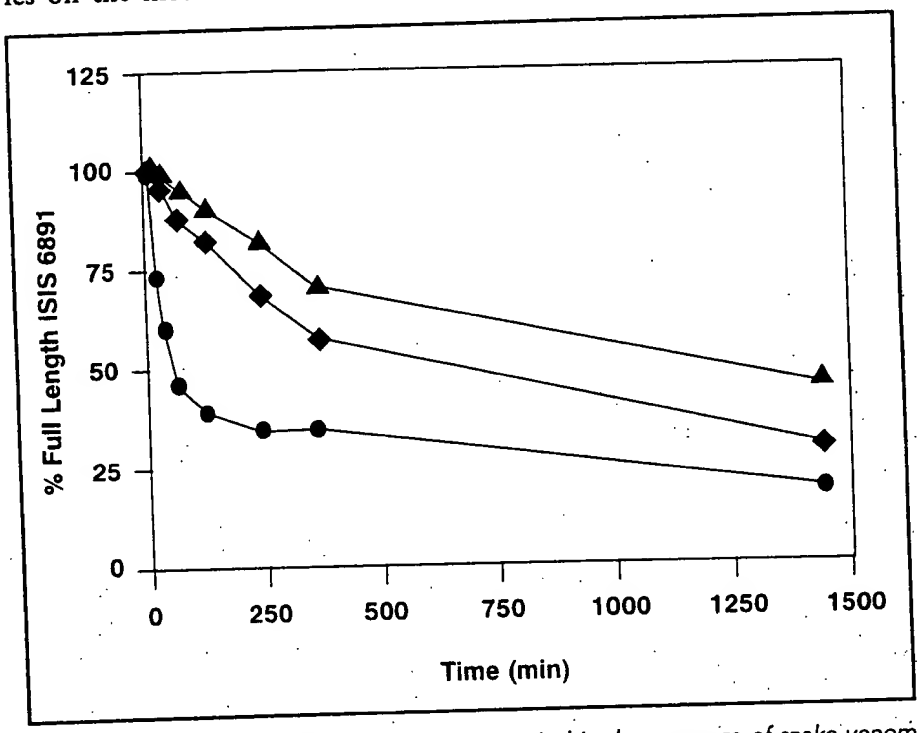

Fig. 5.1. Stability of ISIS 6981 over a 24 hour period in the presence of snake venom phosphodiesterase I. Each point represents the mean of three single samples assayed in three separate experiments. Standard error for all points was less than 2.5% (●) 0.01 μM; (◇), 0.1 μM; (◆), 1.0 μM.

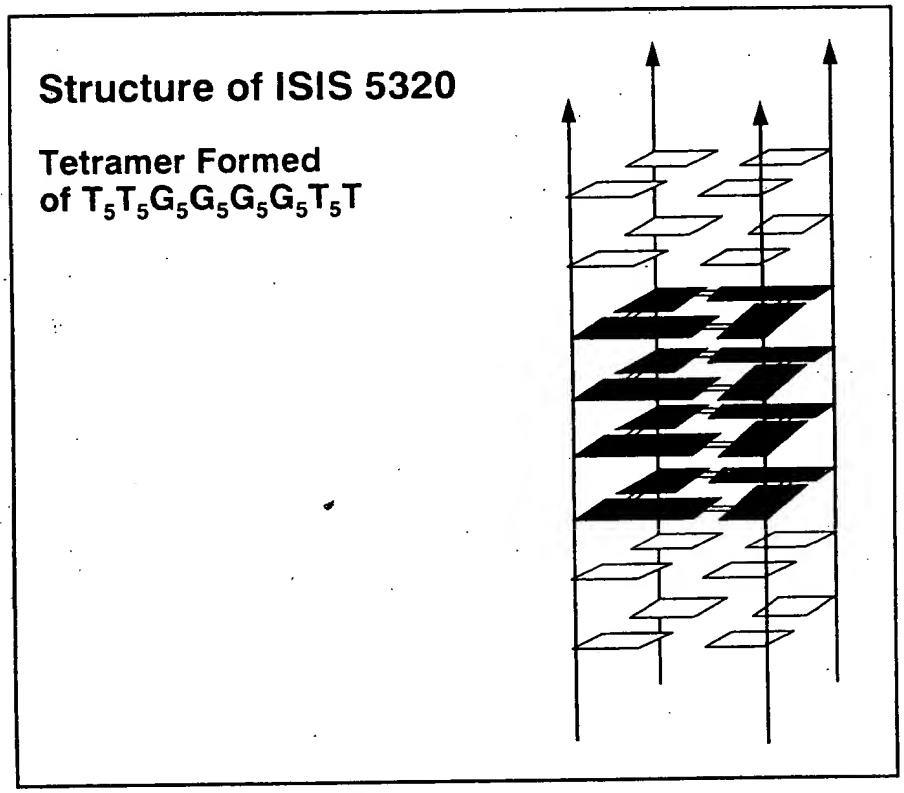

Fig. 5.2. The structure of ISIS 5320.

PHARMACOKINETIC PROPERTIES

To study the pharmacokinetics of phosphorothioate oligonucleotides, a variety of labeling techniques have been used. In some cases, 3' or 5'^{32}P end-labeled or fluorescently-labeled oligonucleotides have been used in in vitro and in vivo studies. These are probably less satisfactory than internally-labeled compounds because terminal phosphates are rapidly removed by phosphatases and fluorescently-labeled oligonucleotides have physico-chemical properties that differ from the unmodified oligonucleotides. Consequently, either uniformly ^{35}S-labeled or base-labeled phosphorothioates have been most extensively used (Fig. 5.3). In our laboratories, a tritium exchange method that labels a slowly exchanging proton at the C8 position in purines was developed and proved to be quite useful (Graham et al, 1993). Very recently, a method that added radioactive methyl groups via S-adenosyl methionine has also been successfully used (Sands et al, 1994).

Nuclease Stability

The principle metabolic pathway for oligonucleotides is cleavage via endo and exonucleases. Phosphorothioate oligonucleotides, while quite stable to various nucleases (Hoke et al, 1991; Wickstrom, 1986; Campbell et al, 1990) are competitive inhibitors of these same enzymes (Gao et al, 1991; Graham et al, 1994). Consequently, the stability of phosphorothioate oligonucleotides to nucleases is probably a

bit less than initially thought, as high concentrations (that inhibited nucleases) of oligonucleotides were employed in the early studies. Similarly, phosphorothioate oligonucleotides are degraded slowly by cells in tissue culture with a half-life of 12-24 hours (Hoke et al, 1991; Graham et al, 1994) and are slowly metabolized in animals (Cossum et al, 1993). The pattern of metabolites suggests primarily exonuclease activity with perhaps modest contributions by endonucleases. For example, Figure 5.4 shows the metabolic pattern of a 20-mer phosphorothioate oligonucleotide incubated with NHDF cells in vitro (RM Crooke et al, 1994).

In Vitro Cellular Uptake

Phosphorothioate oligonucleotides are taken up by a wide range of cells in vitro (RM Crooke, 1993; RM Crooke, 1991; RM Crooke et al, 1994; Neckers, 1993). In fact, very recently, uptake of phosphorothioate oligonucleotides into a prokaryote, *vibrio parahaemoyticus*, has been reported (Chrissey et al, 1993). Uptake is time and temperature dependent. It is also influenced by cell type, cell-culture conditions, media and sequence, and length of the oligonucleotide (RM Crooke

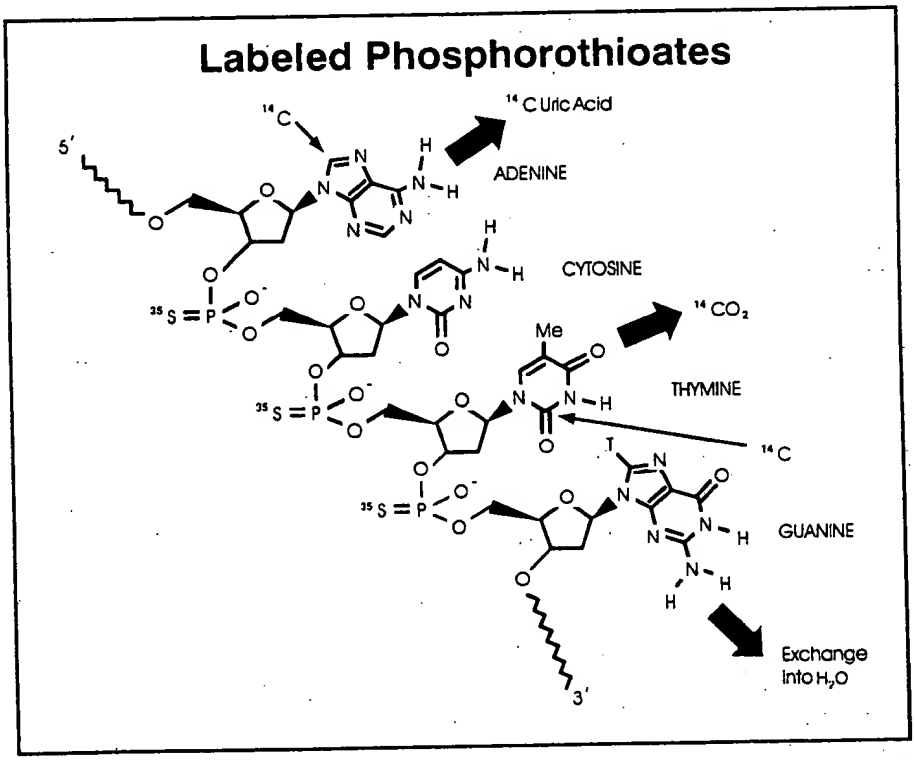

Fig. 5.3. Various labeling methods employed to study the pharmacokinetics of phosphorothioates. The most useful methods have included uniform labeling ^{35}S at all phosphorothioate moieties. Alternatively, bases have been labeled. If labeled at the 2' of pyrimidines with ^{14}C, the ultimate metabolite, CO_2 can be followed in expired air. Alternatively, slowly exchangeable protons at the C8 positions of purines can be labeled with tritium.

et al, 1994). No obvious correlation between the lineage of cells, whether the cells are transformed or whether the cells are virally infected, and uptake has been identified (RM Crooke et al, 1994). Nor are the factors that result in differences in uptake of different sequences of oligonucleotide understood. Although several studies have suggested that receptor-mediated endocytosis may be a significant mechanism of cellular

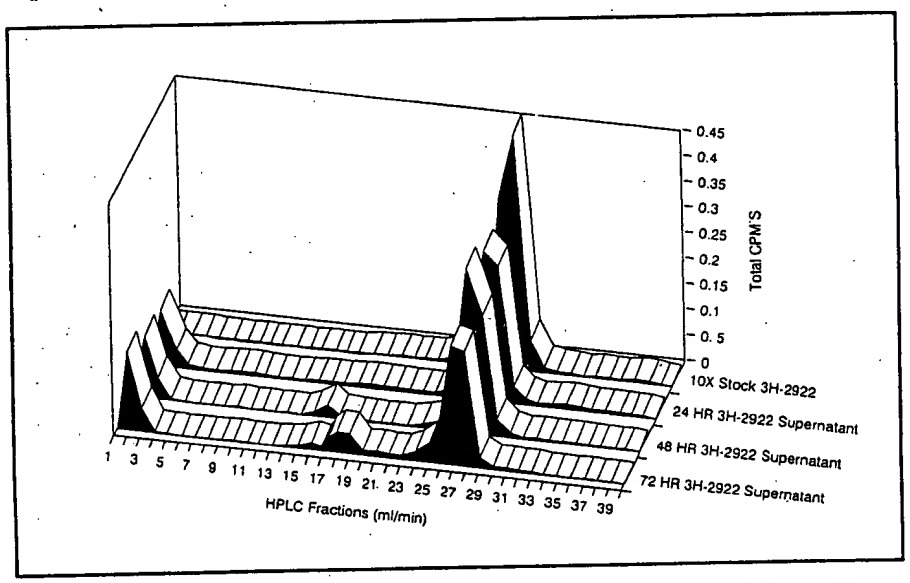

Fig. 5.4A. Stability of ^{3}H-ISIS 2922 in cellular incubation media for 24, 48 and 72 hours. The profile at each time point represents the percentage of the total measured cpms derived from anion exchange HPLC-separated fractions analyzed by LSC counting. 10X stock represents the unincubated starting material.

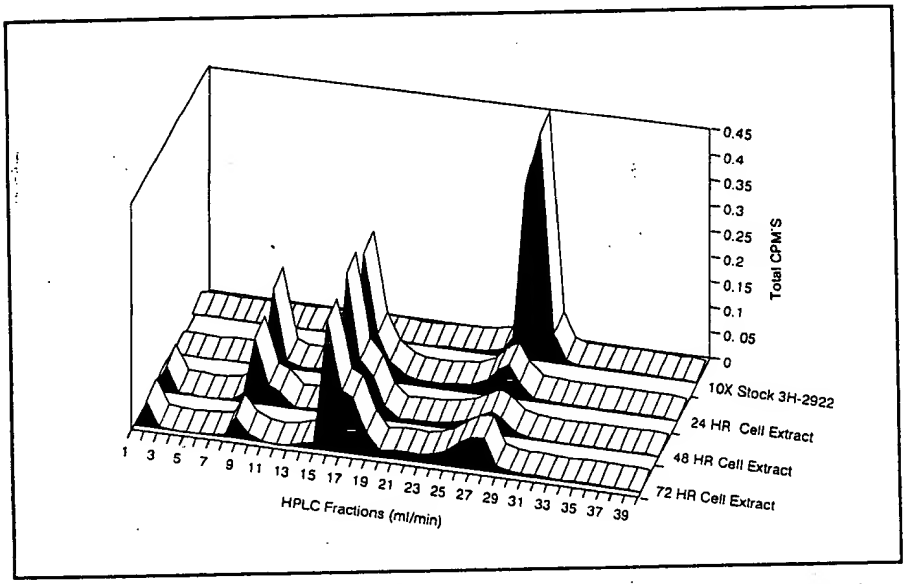

Fig. 5.4B. Stability of ^{3}H-ISIS 2922 extracted from NHDF cells after 24, 48 and 72 hour incubation. The profile at each time point represents the percentage of the total measured cpms derived from anion exchange HPLC separated fractions analyzed by LSC counting. 10X stock represents the unincubated starting material.

uptake (Loke et al, 1989), the data are not yet compelling enough to conclude that receptor-mediated endocytosis accounts for a significant portion of the uptake in most cells.

Numerous studies have shown that phosphorothioate oligonucleotides distribute broadly in most cells once taken up (RM Crooke, 1993; RM Crooke et al, 1994). Again, however, significant differences in subcellular distribution between various types of cells have been noted.

Cationic lipids have been used to enhance uptake of phosphorothioate oligonucleotides in cells that take up little oligonucleotide in vitro (Bennett et al, 1993; Bennett et al, 1992). Again, however, there are substantial variations from cell type to cell type.

In Vivo Pharmacokinetics

Phosphorothioate oligonucleotides bind to serum albumin and α-2 macroglobulin. The apparent affinity for albumin is quite low (250-400 μM) and comparable to the low affinity binding observed for a number of drugs, e.g., aspirin, penicillin (Joos & Hall, 1969). Serum protein binding, therefore, provides a repository for these drugs and prevents rapid renal excretion. As serum protein binding is saturable, at higher doses, intact oligomer may be found in urine (Agrawal et al, 1991; Iversen, 1991). Studies in our laboratory suggest that in rats, IV doses of 15-20 mg/kg saturate the serum protein binding capacity (Leeds, unpublished data).

Phosphorothioate oligonucleotides are rapidly and extensively absorbed after parenteral administration. For example, in rats, after an intradermal dose 3.6 mg/kg of ^{14}C-ISIS 2105, a 20-mer phosphorothioate, approximately 70% of the dose was absorbed within 4 hours and total systemic bioavailability was in excess of 90% (Cossum et al, 1994). After intradermal injection in man, absorption of ISIS 2105 was similar to that observed in rats (Crooke et al, 1994).

Distribution of phosphorothioate oligonucleotides from blood after absorption or intravenous administration is extremely rapid. We have reported distribution half lives of less than one hour (Cossum et al, 1993; Cossum et al, 1994), and similar data have been reported by others (Agrawal et al, 1991; Iversen, 1991). Blood and plasma clearance is multi-exponential with a terminal elimination half life from 40-60 hours in all species except man. In man, the terminal elimination half-life may be somewhat longer (Crooke et al, 1994).

Phosphorothioates distribute broadly to all peripheral tissues. Liver, kidney, bone marrow, skeletal muscle and skin accumulate the highest percentage of a dose, but other tissues display small quantities of drug (Cossum et al, 1993; Cossum et al, 1994). No evidence of significant penetration of the blood brain barrier has been reported. The rates of incorporation and clearance from tissues vary as a function of the organ studied, with liver accumulating drug most rapidly (20% of a dose within 1-2 hours) and other tissues accumulating drug more slowly. Similarly, elimination of drug is more rapid from liver than any other tissue, e.g., terminal half life from liver: 62 hours; from renal medulla: 156 hours.

At relatively low doses, clearance of phosphorothioate oligonucleotides is due primarily to slow metabolism (Cossum et al, 1993; Iversen, 1991; Cossum et al, 1994). Metabolism is mediated by exo and endonucleases that result in shorter oligonucleotides and, ultimately, nucleosides that are degraded by normal metabolic pathways. Although no direct evidence of base excision or modification has been reported, these are theoretical possibilities that may occur. In one study, a larger molecular weight radioactive material was observed in urine, but not fully characterized (Agrawal et al, 1991). Clearly, the potential for conjugation reactions and extension of oligonucleotides via these drugs serving as primers for polymerases must be explored in more detail. In a very thorough study, a pair of 20 nucleotide phosphodiester and phosphorothioate oligonucleotides were administered intravenously at a dose of 6 mg/kg to mice. The oligonucleotides were internally labeled with ^3H-CH_3 by methylation of an internal deoxycytidine residue using Hha1 methylase and S-[^3H] adenosyl methionine (Sands et al, 1994). The observations for the phosphorothioate oligonucleotide were entirely consistent with those made in our studies. Additionally, in this paper, autoradiographic analyses showed drug in renal cortical cells (Sands et al, 1994).

One study of prolonged infusions of a phosphorothioate oligonucleotide to human beings has been reported (Bayever et al, 1993). In this study, five patients with leukemia were given ten-day intravenous infusions at a dose of 0.05 mg/kg/hour. Elimination half lives reportedly varied from 5.9 to 14.7 days. Urinary recovery of radioactivity was reported to be 30-60% of the total dose, with 30% of the radioactivity being intact drug. Metabolites in urine included both higher and lower molecular weight compounds. Obviously, these data differ from observations in other studies. At present, the data are insufficient to determine if the pharmacokinetics of prolonged intravenous infusions are truly substantially different from the pharmacokinetic behavior of intravenous bolus injections.

We have also performed oral bioavailability experiments in rodents treated with an H2 antagonist to avoid acid-mediated depurination or precipitation. In these studies, very limited (<10%) bioavailability was observed.

Figure 5.5 compares the plasma clearance of ISIS 2302, a 20-mer phosphorothioate, in mice and monkeys. It demonstrates that there are minimal species-associated differences in plasma clearance. Nor are there significant differences in distribution. However, slight differences in metabolic rate have been noted with phosphorothioates of different sequences.

In summary, pharmacokinetic studies of several phosphorothioates demonstrate that they are well absorbed from parenteral sites, distribute broadly to all peripheral tissues, do not cross the blood brain barrier and are eliminated primarily by slow metabolism. In short, once a day or every other day systemic dosing should be feasible. Although the similarities between oligonucleotides of different sequences are far greater than the differences, additional studies are required before determining whether there are subtle effects of sequence on the pharmacokinetic profile of this class of drugs.

PHARMACOLOGICAL PROPERTIES

Molecular Pharmacology

Antisense oligonucleotides are designed to bind to RNA targets via Watson-Crick hybridization. As RNA can adopt a variety of secondary structures via Watson-Crick hybridization, one useful way to think of antisense oligonucleotides is as competitive antagonists for self-complementary regions of the target RNA. Obviously, creating oligonucleotides with the highest affinity per nucleotide unit is pharmacologically important, and a comparison of the affinity of the oligonucleotide to a complementary RNA oligonucleotide is the most sensible comparison. In this context, phosphorothioate oligodeoxynucleotides are relatively competitively disadvantaged as the affinity per nucleotide unit of oligomer is less than RNA (>-2.0°T_m per unit) (Cook, 1993). This results in a requirement of at least 15-17 nucleotides in order to have sufficient affinity to produce biological activity (Monia et al, 1992).

Although multiple mechanisms by which an oligonucleotide may terminate the activity of an RNA species to which it binds are possible, examples of biological activity have been reported for only three. Antisense oligonucleotides have been reported to inhibit RNA splicing (Kulka et al, 1989), and translation (Agrawal et al, 1988) of mRNA and to induce degradation of RNA by RNase H (Chiang et al, 1991). Without question, the mechanism that has resulted in the most potent compounds and is best understood is RNase H activation. To

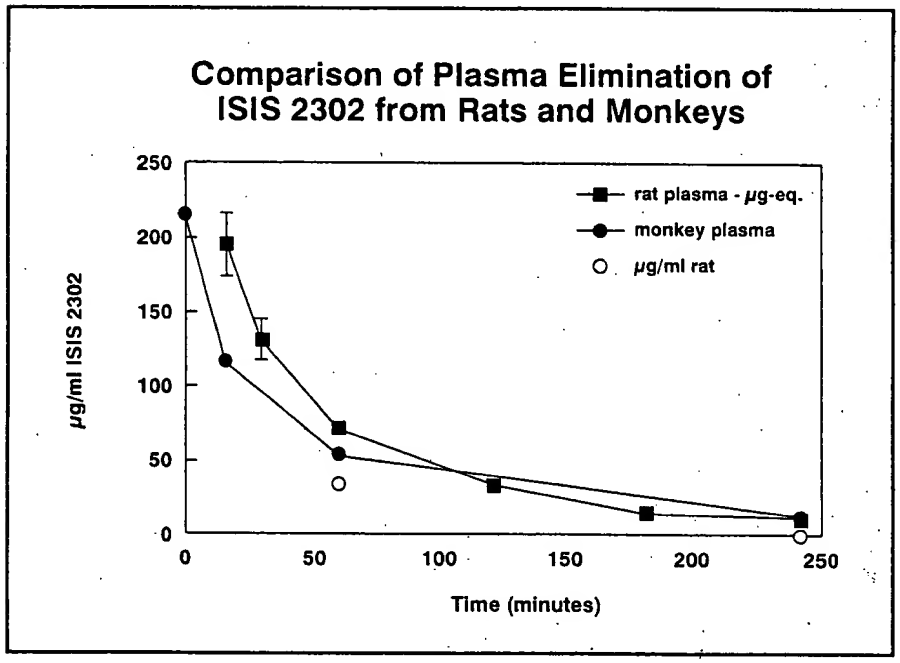

Fig. 5.5. Plasma clearance of ^{14}C ISIS 2302 in rats and monkeys. A dose of approximately 4 mg/kg IV was administered intravenously to rats or monkeys and plasma levels of radioactivity and intact drug were measured as previously described (Cossum et al, 1994).

serve as a substrate for RNase H, a duplex between RNA and a "DNA-like" oligonucleotide is required. Specifically, a sugar moiety in the oligonucleotide that induces a duplex conformation equivalent to that of a DNA-RNA duplex and a charged phosphate are required (for review, see Mirabelli and Crooke, 1993). Thus, phosphorothioate oligodeoxynucleotides are expected to induce RNase H-mediated cleavage of the RNA when bound. As will be discussed later, many chemical approaches that enhance the affinity of an oligonucleotide for RNA result in duplexes that are no longer substrates for RNase H.

Selection of sites at which optimal antisense activity may be induced in a RNA molecule is complex, dependent on terminating mechanism and influenced by the chemical class of the oligonucleotide. Each RNA appears to display unique patterns of sites of sensitivity. Within the phosphorothioate oligodeoxynucleotide chemical class, studies in our laboratory have shown antisense activity can vary from undetectable to 100% by shifting an oligonucleotide by just a few bases in the RNA target (Crooke, 1992; Chiang et al, 1991; Bennett & Crooke, 1994, chapter 3 of this volume). Although significant progress has been made in developing general rules that help define potentially optimal sites in RNA species, to a large extent, this remains an empirical process that must be performed for each RNA target and every new chemical class of oligonucleotides.

Phosphorothioates have also been shown to have effects inconsistent with the antisense mechanism for which they were designed. Some of these effects are due to sequence and structure specific, as well as non-specific interactions with proteins. These effects are particularly prominent in in vitro tests for antiviral activity as often high concentrations of cells, viruses and oligonucleotides are coincubated (Azad et al, 1993; Wagner et al, 1993). Human immune deficiency virus (HIV) is particularly problematic as many oligonucleotides bind to the gp120 protein and other proteins of the virus (Wyatt et al, 1994). In addition to protein interactions, other factors, such as overrepresented sequences of RNA and unusual structures that may be adopted by oligonucleotides, can contribute to unexpected results (Wyatt et al, 1994).

Given the variability in cellular uptake of oligonucleotides, the variability in potency as a function of binding site in an RNA target and potential non-antisense activities of oligonucleotides, careful evaluation of dose-response curves and clear demonstration of the antisense mechanism are required before drawing conclusions from in vitro experiments. Nevertheless, numerous well-controlled studies have been reported in which antisense activity was conclusively demonstrated. As many of these studies have been reviewed previously (Crooke, 1992; Crooke & Lebleu, 1993; Crooke, 1994; Crooke, 1993; Nagel et al, 1993; Stein & Cheng, 1993), suffice it to say that antisense effects of phosphorothioate oligodeoxynucleotides against a variety of targets are well documented.

In Vivo Pharmacological Activities

Table 5.1 summarizes the published reports (abstracts and full publications) demonstrating in vivo activity of oligonucleotides. Local

effects have been reported for phosphorothioate and methylphosphonate oligonucleotides. A phosphorothioate oligonucleotide designed to inhibit c-myb production and applied locally was shown to inhibit intimal accumulation in the rat carotid artery (Simons et al, 1992). In this study, a Northern blot showed a significant reduction in c-myb RNA in animals treated with the antisense compound, but no effect by a control oligonucleotide. Similar effects were reported for phosphorothioate oligodeoxynucleotides designed to inhibit cyclin-dependent kinases (CDC-2 and CDK-2). Again, the antisense oligonucleotide inhibited intimal thickening and cyclin-dependent kinase activity, while a control oligonucleotide had no effect (Abe et al, 1994). Additionally, local administration of a phosphorothioate oligonucleotide designed to inhibit N-myc resulted in reduction in N-myc expression and slower growth of a subcutaneously transplanted human tumor in nude mice (Whitesell et al, 1991).

Local antitumor effects of phosphorothioate oligodeoxynucleotides have also been reported. An antisense oligonucleotide designed to inhibit the expression of the p120 protein was shown to inhibit the growth of a human tumor transplanted intraperitoneally in nude mice when the compound was administered intraperitoneally (Perlaky et al, 1993).

Antisense oligonucleotides administered intraventricularly have been reported to induce a variety of effects in the central nervous system. Intraventricular injection of antisense oligonucleotides to neuropeptide-y-y1 receptors reduced the density of the receptors and resulted in behavioral signs of anxiety (Wahlestedt et al, 1993). Similarly, an antisense oligonucleotide designed to bind to NMDA-R1 receptor channel RNA inhibited the synthesis of these channels and

Table 5.1. Reported activities of antisense drugs in animals

Target	Animal	Reference
HSV-1	Mouse	Kulka et al, 1989
HSV-1	Mouse	Brandt et al, 1991
Tick-Borne Encephalitis Virus	Mouse	Vlassov, 1989
p120 Oncogene	Mouse	Perlaky et al, 1993
c-myb	Rat	Simons et al, 1992
Interleukin 1	Mouse	Burch & Mahan, 1991
NF-KB	Mouse	Kitajima et al, 1992
CDC-2 and CDK-2	Rat	Abe et al, 1994
N-myc	Mouse	Whitesell et al, 1991
Y-Y1 Receptors	Rat	Wahlestedt et al, 1993
NMDA-R1 Receptor Channel	Rat	Wahlestedt et al, 1993
Symptosomal-Associated Protein 25	Rat	Osen-Sand et al, 1993
NF-KB	Mouse	Higgins et al, 1993
Intercellular Adhesion Molecule 1	Mouse	Stepkowski et al, 1994
Protein Kinase C-α	Mouse	Dean & McKay, 1994
Philadelphia[1] Leukemia	Mouse	Skorski et al, 1994
MYB	Mouse	Hijiya et al, 1994

reduced the volume of focal ischemia produced by occlusion of the middle cerebral artery in rats (Wahlestedt et al, 1993).

Injection of antisense oligonucleotides to synaptosomal-associated protein-25 into the vitreous body of rat embryos reduced the expression of the protein and inhibited neurite elongation by rat cortical neurons (Osen-Sand et al, 1993).

In addition to local and regional effects of antisense oligonucleotides, a growing number of well-controlled studies have demonstrated systemic effects of phosphorothioate oligodeoxynucleotides. Expression of interleukin-1 in mice was inhibited by systemic administration of antisense oligonucleotides (Burch & Mahan, 1991). Oligonucleotides to the NF-kB p65 subunit administered intraperitoneally at 40 mg/kg every three days slowed tumor growth in mice transgenic for the human T-cell leukemia viruses (Kitajima et al, 1992). Similar results with other antisense oligonucleotides were shown in another in vivo tumor model after either prolonged subcutaneous infusion or intermittent subcutaneous injection (Higgins et al, 1993).

Two recent reports further extend the studies of phosphorothioate oligonucleotides as anti-tumor agents in mice. In one study, a phosphorothioate oligonucleotide directed to inhibition of the BCR-ABL oncogene was administered at a dose of 1 mg/day for nine days intravenously to immunodeficient mice injected with human leukemic cells. The drug was shown to inhibit the development of leukemic colonies in the mice and to selectively reduce BCR-ABL RNA levels in peripheral blood lymphocytes, spleen, bone marrow, liver, lungs and brain (Skorski et al, 1994). In the second study, a phosphorothioate oligonucleotide antisense to the protooncogene myb, inhibited the growth of human melanoma in mice. Again, myb mRNA levels appeared to be selectively reduced (Hijiya et al, 1994).

Thus, there is a growing body of evidence suggesting that antisense oligonucleotides (in most cases, phosphorothioate oligodeoxynucleotides) can produce local, regional and systemic effects at non-toxic doses in vivo. Although proof of mechanism of action is difficult, in most cases, studies with control oligonucleotides demonstrate that the effects are consistent with the proposed antisense mechanism. In the important series of studies by Dean and colleagues (Dean & McKay, 1994), an antisense mechanism and isotype selectivity have been conclusively demonstrated after systemic administration of an antisense oligonucleotide designed to inhibit protein kinase C-α (PKC-α) in mice.

All of these data suggest that phosphorothioate oligodeoxynucleotides may have therapeutic potential.

TOXICOLOGICAL PROPERTIES

In Vitro

In our laboratory, we have evaluated the toxicities of scores of phosphorothioate oligodeoxynucleotides in a significant number of cell lines in tissue culture. As a general rule, no significant cytotoxicity is induced at concentrations below 100 μM oligonucleotide. Additionally, with a few exceptions, no significant effect on macromolecular

synthesis is observed at concentrations below 100 μM (RM Crooke, 1993; RM Crooke, 1993).

Polynucleotides and other polyanions have been shown to cause release of cytokines (Colby, 1971). Also, bacterial DNA species have been reported to be mitogenic for lymphocytes in vitro (Messina et al, 1991). Furthermore, oligodeoxynucleotides (30-45 nucleotides in length) were reported to induce interferons and enhance natural killer cell activity (Kuramoto et al, 1992). In the latter study, the oligonucleotides that displayed natural killer cell (NK)-stimulating activity contained specific palindromic sequences and tended to be guanosine rich. Collectively, these observations indicate that nucleic acids may have broad immunostimulatory activity.

It has been shown that phosphorothioate oligonucleotides stimulate B-lymphocyte proliferation in a mouse splenocyte preparation (analogous to bacterial DNA) (Pisetsky & Reich, 1993), and the response may underlie the observations of lymphoid hyperplasia in the spleen and lymph nodes of rodents caused by repeated administration of these compounds (see below). We also have evidence of enhanced cytokine release by immunocompetent cells when exposed to phosphorothioates in vitro (RM Crooke et al, 1994).

Genotoxicity

As with any new chemical class of therapeutic agents, concerns about genotoxicity cannot be dismissed as little in vitro testing has been performed and no data from long-term studies of oligonucleotides are available. Clearly, given the limitations in our understanding about the basic mechanisms that might be involved, empirical data must be generated. We have performed mutagenicity studies on two phosphorothioate oligonucleotides, ISIS 2105 and ISIS 2922, and found them to be non-mutagenic at all concentrations studied (Crooke, 1994).

Two mechanisms of genotoxicity that may be unique to oligonucleotides have been considered. One possibility is that an oligonucleotide analog could be integrated into the genome and produce mutagenic events. Although integration of an oligonucleotide into the genome is conceivable, it is likely to be extremely rare. For most viruses, viral DNA integration is itself a rare event and, of course, viruses have evolved specialized enzyme-mediated mechanisms to achieve integration. Moreover, preliminary studies in our laboratory have shown that phosphorothioate oligodeoxynucleotides are generally poor substrates for DNA polymerases, and it is unlikely that enzymes such as integrases, gyrases and topoisomerases (that have obligate DNA cleavage as intermediate steps in their enzymatic processes) will accept these compounds as substrates. Consequently, it would seem that the risk of genotoxicity due to genomic integration is no greater and probably less than that of other potential mechanisms, for example, alteration of the activity of growth factors, cytokine release, non-specific effects on membranes that might trigger arachidonic acid release or inappropriate intracellular signalling. Presumably, new analogs that deviate significantly more from natural DNA would be even less likely to be integrated.

A second concern that has been raised about possible genotoxicity is the risk that oligonucleotides might be degraded to toxic or carcinogenic metabolites. However, metabolism of phosphorothioate oligodeoxynucleotides by base excision would release normal bases, which presumably would be non-genotoxic. Similarly, oxidation of the phosphorothioate backbone to the natural phosphodiester structure would also yield non-mutagenic (and probably non-toxic) metabolites. Finally, it is possible that phosphorothioate bonds could be hydrolyzed slowly, releasing nucleoside phosphorothioates that presumably would be rapidly oxidized to natural (non-toxic) nucleoside phosphates. However, oligonucleotides with modified bases and/or backbones may pose different risks.

In Vivo

The acute LD_{50} dose in mice of all phosphorothioates tested to date is in excess of 500 mg/kg (Kornbrust, unpublished observations). Although there may be differences between different oligonucleotide sequences and the LD_{50} may be influenced by route of administration, these factors appear to result in minimal variation. Several phosphorothioate oligodeoxynucleotides have been studied for potential fetal toxicities and, to date, no significant adverse effects have been noted (Kornbrust, unpublished observations; Guadette et al, 1993).

Although there is no evidence of antigenicity or induction of delayed type hypersensitivity in animals or humans given multiple doses of phosphorothioate oligonucleotides (Crooke, 1993; Kornbrust, unpublished observations), multiple doses of these drugs clearly affect the immune system in animals, with rats being the most sensitive species. The manifestations of these toxicities that are observed at lowest doses are increases in spleen weight, production of IgM and IgG and expansion of B-cell populations in spleens (Kornbrust, unpublished observations; Branda et al, 1993). Although the sequence of the oligonucleotides can affect the dose at which these effects are produced and an antisense sequence designed to inhibit the 65 kd subunits of NF-kB was reported to be devoid of such effects (McIntyre et al, 1993), in our experience, all thoroughly tested phosphorothioate oligodeoxynucleotides induced these effects. As a general rule, the doses required to induce these effects have been substantially greater than those demonstrating pharmacologic activity.

In monkeys, several phosphorothioate oligodeoxynucleotides have been shown to cause acute hypotensive events (Kornbrust, unpublished observations; Cornish et al, 1993). These effects are transient, if managed appropriately, relatively uncommon and typically appear to occur in response to the first dose given to an animal. Recent studies suggest that one mechanism responsible for this may be related to complement activation, and that this toxicity can be avoided by giving intravenous infusions rather than bolus injections. We have evaluated the mechanisms by which phosphorothioate oligonucleotides might induce complement activation and phosphorothioates appear to affect the alternative pathway. In large part, the effects of phosphorothioates are

similar to those associated with other polyanions, such as heparin. Complement regulatory factors, such as Factor H and D, appear to be most influenced by these drugs. Based on predictions about studies on animal models, toxicological studies in monkeys and unpublished reports studies on systemically administered with phosphorothioates currently in the clinic, the therapeutic index relative to this potential toxicity would appear to be likely to be acceptable.

Very recently, we have also noted prolongation of prothrombin, partial thromboplastin and bleeding tissues in monkeys. Again, these effects are plasma concentration dependent and appear to occur at doses that are sufficient to saturate serum albumin binding sites. The effects on partial thromboplastin time are much more pronounced than those on prothrombin time. The effects appear to be primarily on the extrinsic pathway with clear inhibition of thrombin activity demonstrated. No effects on Factors VIII-XI have been observed. Again, based on prediction from studies in monkeys, the doses likely to produce this toxicity seem substantially greater than predicted therapeutic doses. Clearly, only carefully controlled clinical trials will define the human toxicities and therapeutic indices of these drugs.

Clinical Activities

To date, we have studied several hundred patients given multiple doses of two phosphorothioate oligonucleotides and encountered no significant toxicities. Other oligonucleotides are being studied in human beings by other groups and, to date, no significant or dose-related toxicities have been encountered.

ISIS 2922, a 20-mer phosphorothioate designed to inhibit cytomegalovirus, has been administered intravitreally to patients with advanced AIDS and advanced drug-resistant CMV retinitis and showed impressive antiviral activity (Crooke, 1994).

In this study, three dose groups were studied in patients that had advanced CMV retinitis and had failed ganciclovir and/or foscarnet therapy. The median CD4 count in these patients was 4 so they were extremely immunocompromised. The median time from diagnosis of CMV retinitis was 10 months. Two patients were treated in the 2 μM dose group and failed. Three of four eyes treated at 4 μM and six of eight eyes at 8 μM responded. The responses were rapid, long lasting and substantial. The adverse events observed were increased inflammation (Palestine et al, 1994).

ISIS 2105 is a 20-mer phosphorothioate oligonucleotide designed to inhibit the replication of human papilloma viruses 6 and 11, the viruses responsible for genital warts. Pilot ascending dose multiple dose studies as primary therapy of genital warts have shown the drug locally and systemically well tolerated after intradermal administration. At doses of 2.5 mg/wart twice weekly and greater, the drug has been associated with resolution of genital warts. In a pivotal placebo controlled trial of patients treated by surgical removal of the warts and administration of single doses of 0.3 or 1.0 mg/wart ISIS 2105 at the time of surgery, reductions in wart recurrence were observed that were

not statistically significant, but suggestive of antiviral activity were observed.

Development of ISIS 2922 is proceeding with Phase III trials in patients with CMV retinitis. Development of ISIS 2105 is proceeding with a multiple dose surgical adjuvant Phase II trial designed to confirm its activity and evaluate its potential utility.

CONCLUSIONS

Phosphorothioate oligonucleotides have perhaps outperformed many expectations. They display attractive parenteral pharmacokinetic properties. They have produced potent systemic effects in a number of animal models and, in many experiments, the antisense mechanism has been directly demonstrated as the hoped-for selectivity. Further, these compounds appear to display satisfactory therapeutic indices for many indications.

Nevertheless, phosphorothioates clearly have significant limits (Table 5.2). Pharmacodynamically, they have relatively low affinity per nucleotide unit. This means that longer oligonucleotides are required for biological activity and that invasion of many RNA structures may not be possible. At higher concentrations, these compounds inhibit RNase H as well. Thus, the higher end of the pharmacologic dose response curve is lost. Pharmacokinetically, phosphorothioates do not cross the blood brain barrier, are not significantly orally bioavailable and may display dose-dependent pharmacokinetics. Toxicologically, clearly the release of cytokines, activation of complement and interference with clotting will pose dose limits if they are encountered in the clinic.

As several clinical trials are in progress with phosphorothioates and others will be initiated shortly, we shall soon have more definitive information about the activities, toxicities and value of this class of antisense drugs in human beings.

Table 5.2. Phosphorothioate oligonucleotides

Limits
• Pharmacodynamic
• Low affinity per nucleotide unit
• Inhibition of RNase H at high concentrations
• Pharmacokinetic
• Limited bioavailability
• Limited blood brain barrier penetration
• Dose-dependent pharmacokinetics
• Toxicologic
• Release of cytokines
• Complement associated effects on blood pressure?
• Clotting effects

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